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13. ABSTRACT (Maximum 200 Words) In the present work we demonstrate that estradiol and its metabolites mainly 4-OH estradiol are able to induce transformation phenotypes in the human breast epithelial cells (HBEC) MCF-10F. MCF10F cells is ER α negative, although, they ER- β positive that could indicate that the response of the cells to growth and form colonies in agar methocel could be mediated by this receptor. However, the Invasion phenotype is not modified when the cells are treated in presence of tamoxifen or ICI, suggesting that other pathways may be involved. With the data presently available the direct effect of 4-OH-E2 support the concept that metabolic activation of estrogens mediated by various cytochrome P450 complexes, generating through this pathway reactive intermediates that elicit direct genotoxic effects leading to transformation. This assumption was confirmed when we found that all the transformation phenotypes induced by 4-OH-E2 were not abrogated when this compound was used in presence of the pure antiestrogenic ICI. We have detected loss of heterozygosity (LOH) in ch13q12.2-12.3 (D13S893) and in ch17q21.1 that has been reported in primary breast cancer, that the changes are similar to those induced by the chemical carcinogen (BP) and that the genomic changes were not abrogated by antiestrogens.			
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A-INTRODUCTION

Estradiol-17 β is biologically the most active estrogen in breast tissue. Circulating estrogens are mainly originated from ovarian steroidogenesis in premenopausal women and peripheral aromatization of ovarian and adrenal androgens in postmenopausal women (1). The importance of ovarian steroidogenesis in the genesis of breast cancer is highlighted by the fact that occurring naturally or induced early menopause prior to age 40 significantly reduces the risk of developing breast cancer (1). However, the uptake of estradiol-17 β from the circulation does not appear to contribute significantly to the total content of estrogen in breast tumors, since the majority of estrogen present in the tumor tissues is derived from *de novo* biosynthesis (1). In fact, the concentrations of estradiol-17 β in breast cancer tissues do not differ between premenopausal and postmenopausal women, even though plasma levels of estradiol-17 β decrease by 90% following menopause (2). This phenomenon might be explained by the observation that enzymatic transformations of circulating precursors in peripheral tissues contribute 75% of estrogens in premenopausal women and almost 100% in postmenopausal women (3,4), the data that highlight the importance of *in situ* metabolism of estrogens. Three main enzyme complexes that are involved in the synthesis of biologically active estrogen (i.e. estradiol-17 β) in the breast are: 1) aromatase that converts androstenedione to estrone, 2) estrone sulfatase that hydrolyses the estrogen sulfate to estrone, and 3) estradiol-17 β hydroxysteroid dehydrogenase that preferentially reduces estrone to estradiol-17 β in tumor tissues (5, 6).

Although 67% of breast cancers are manifested during the postmenopausal period, a vast majority, 95%, is initially hormone-dependent (1). This indicates that estrogens play a crucial role in their development and evolution (7-9). However, it is still unclear whether estrogens are carcinogenic to the human breast. There are three mechanisms that have been considered to be responsible for the carcinogenicity of estrogens: receptor-mediated hormonal activity, which has generally been related to stimulation of cellular proliferation, resulting in more opportunities for accumulation of genetic damages leading to carcinogenesis (10), a cytochrome P450-mediated metabolic activation, which elicits direct genotoxic effects by increasing mutation rates (11,2), and the induction of aneuploidy by estrogen (13-20). There is also evidence that estrogen compromises the DNA repair system and allows accumulation of lesions in the genome essential to estrogen-induced tumorigenesis (21).

A-i Receptor mediated pathway.

The receptor-mediated activity of estrogen is generally related to induction of expression of the genes involved in the control of cell cycle progression and growth of human breast epithelium. The biological response to estrogen depends upon the local concentrations of the active hormone and its receptors. The level of ER expression is higher in breast cancer patients than in control subjects and is related to breast cancer risk in postmenopausal women (22). It has been suggested that overexpression of ER in normal human breast epithelium may augment estrogen responsiveness and hence the risk of breast cancer (22). The proliferative activity and the percentage of ER α -positive cells are highest in Lob 1 in comparison with the various lobular structures composing the normal breast. These findings provide a mechanistic explanation for the higher susceptibility of these structures to be transformed by chemical carcinogens *in vitro* (23,24), supporting as well the observations that Lob 1 are the site of origin of ductal carcinomas (25).

The presence of ER α -positive and ER α -negative cells with different proliferative activity in the normal human breast may help to elucidate the genesis of ER α -positive and ER α -negative breast cancers (26,

27). It has been suggested that either ER α -negative breast cancers result from the loss of the ability of the cells to synthesize ER α during clinical evolution of ER α -positive cancers, or that ER α -positive and ER α -negative cancers are different entities (27,28). Based on these observations, it is postulated that Lob 1 contain at least three cell types, ER α -positive cells that do not proliferate, ER α -negative cells that are capable of proliferating, and a small proportion of ER α -positive cells that can proliferate as well (29). Therefore, estrogen might stimulate ER α -positive cells to produce a growth factor that in turn stimulates neighboring ER α -negative cells capable of proliferating (29). In the same fashion, the small proportion of cells that are ER α -positive and can proliferate could be the stem cell of ER α -positive tumors. The possibility exists, as well, that the ER α -negative cells convert to ER α -positive cells (29) or that they express ER- β .

The newly discovered ER β opens another possibility that those cells traditionally considered negative for ER α might be positive for ER β (30-32). It has recently been found that ER β is expressed during the immortalization and transformation of ER-negative human breast epithelial cells (33), supporting the hypothesis of conversion from a negative to a positive receptor cell. The functional role of ER β -mediated estrogen signaling pathways in the pathogenesis of malignant diseases is essentially unknown. In the rats, ER β -mediated mechanisms have been implicated in the upregulation of PgR expression in the dysplastic acini of the dorsolateral prostate in response to treatment of testosterone and estradiol-17 β (34). In the human, ER β has been detected in both normal and cancerous breast tissues or cell lines, and is the predominant ER type in normal breast tissue. Expression of ER β in breast tumors is inversely correlated with the PgR status and variant transcripts of ER β have been observed in some breast tumors (1). ER β and ER α are co-expressed in some breast tumors and a few breast cell lines, suggesting an interesting possibility that ER α and ER β proteins may interact with each other and discriminate between target sequences leading to differential responsiveness to estrogens. In addition, estrogen responses mediated by ER α and ER β may vary with different composition of their co-activators that transmit the effect of ER-ligand complex to the transcription complex at the promotor of target genes (35). Recently, it has been shown that an increase in the expression of ER α with a concomitant reduction in ER β expression occurs during tumorigenesis of the breast (36) and ovary (37), but breast tumors expressing both ER α and ER β are lymph node-positive and tend to be of higher histopathological grade (1). These data suggest a change in the interplay of ER α - and ER β -mediated signal transduction pathways during breast tumorigenesis.

Even though it is now generally believed that alterations in the ER-mediated signal transduction pathways contribute to breast cancer progression toward hormonal independence and more aggressive phenotypes, there is also mounting evidence that a membrane receptor coupled to alternative second messenger signaling mechanisms (38, 39) are operational, and may stimulate the cascade of events leading to cell proliferation. This knowledge suggests that ER α -negative cells found in the human breast may respond to estrogens through this or other pathways. The biological responses elicited by estrogens are mediated, at least in part, by the production of autocrine and paracrine growth factors from the epithelium and the stroma in the breast (40). In addition, evidence has accumulated over the last decade supporting the existence of ER variants, mainly a truncated ER and an exon deleted ER (41). It has been suggested that expression of ER variants may contribute to breast cancer progression toward hormone independence (41). Although more studies need to be done in this direction, it is clear that the findings that in the normal breast the proliferating and steroid hormone receptor positive cells are different open new possibilities for clarifying the mechanisms through which estrogens might act on the proliferating cells to initiate the cascade of events leading to cancer.

A-ii- Oxidative metabolism of estrogen.

There is evidence that oxidative catabolism of estrogens mediated by various cytochrome P450 (CYP) complexes constitutes a pathway of their metabolic activation and generates reactive free radicals and intermediate metabolites reactive intermediates that can cause oxidative stress and genomic damage directly (11, 12). Estradiol-17 β and estrone, which are continuously interconverted by estradiol-17 β hydroxysteroid dehydrogenase (or 17 β -oxidoreductase), are the two major endogenous estrogens. They are generally metabolized via two major pathways: hydroxylation at C-16 α position and at the C-2 or C-4 positions (42, 43). The carbon position of the estrogen molecules to be hydroxylated differs among various tissues and each reaction is probably catalyzed by various CYP isoforms. For example, in MCF-7 human breast cancer cells, which produce catechol estrogens in culture, CYP 1A1 catalyzes hydroxylation of estradiol-17 β at C-2, C-15 α and C-16 α , CYP 1A2 predominantly at C-2 (1, 44), and a member of the CYP 1B subfamily is responsible for the C-4 hydroxylation of estradiol-17 β . CYP3A4 and CYP3A5 have also been shown to play a role in the 16 α -hydroxylation of estrogens in human (1).

The hydroxylated estrogens are catechol estrogens that will easily be autoxidized to semiquinones and subsequently quinones, both of which are electrophiles capable of covalently binding to nucleophilic groups on DNA via a Michael addition and, thus, serve as the ultimate carcinogenic reactive intermediates in the peroxidative activation of catechol estrogens. In addition, a redox cycle consisting of the reversible formation of the semiquinones and quinones of catechol estrogens catalyzed by microsomal P450 and cytochrome P450-reductase can locally generate superoxide and hydroxyl radicals to produce additional DNA damage. Furthermore, catechol estrogens have been shown to interact synergistically with nitric oxide present in human breast generating a potent oxidant that induces DNA strand breakage (1). Steady state concentrations of catechol estrogens are determined by the cytochrome P450-mediated hydroxylations of estrogens and monomethylation of catechols catalyzed by blood-borne catechol *o*-methyltransferase (45, 46). Increased formation of catechol estrogens as a result of elevated hydroxylations of estradiol-17 β at C-4 and C-16 α (1, 47) positions occurs in human breast cancer patients and in women at a higher risk of developing this disease. There is also evidence that lactoperoxidase, present in milk, saliva, tears and mammary glands, catalyzes the metabolism of estradiol-17 β to its phenoxy radical intermediates, with subsequent formation of superoxide and hydrogen peroxide that might be involved in estrogen-mediated oxidative stress (48). A substantial increase in base lesions observed in the DNA of invasive ductal carcinoma of the breast (49) has been postulated to result from the oxidative stress associated with metabolism of estradiol-17 β (48).

The detection of various types of DNA damage induced by estrogen metabolites in cell-free systems or in cells in culture and by parent hormones *in vivo* (50-54) has led to the hypothesis of an additional role of estrogen as mutagen and tumor initiator (55,56). The induction of mutations by estrogens or their metabolites has been demonstrated (57, 58) supporting the hypothesis that estrogens are mutagenic and that metabolic conversion of E₂ to catechol estrogen is required for the induction of such mutations. In addition to mutations, E₂ also induces microsatellite instability. Changes in DNA fragments containing microsatellite repeat sequences have been detected in E₂-induced hamster kidney tumors, in surrounding kidney tissue (59) and in MCF-10F HBEC transformed by E₂ (60). Microsatellite instability is a relatively common genetic modification (61-63), induced by the natural hormone E₂ in cells in culture (109), in Syrian hamster kidney tumors, and in surrounding tissues (59). It has also been detected with high frequency in human vaginal tumors in daughters of women treated with diethylstilbestrol (DES) (64). Microsatellite instability has also been detected in human breast tumors (65-72).

Chemical carcinogens covalently bind to DNA to form two types of adducts: stable ones that remain in DNA unless removed by repair and depurinating ones that are lost from DNA by destabilization of the glycosyl bond (73-74). Evidence that depurinating polycyclic aromatic hydrocarbon-DNA adducts play a major role in tumor initiation (73-75) and that estrogen metabolites form depurinating DNA adducts

strongly indicates that estrogen is an endogenous initiators of cancer (50). Catechol estrogens (CE) are among the major metabolites of estrone (E₁) and estradiol (E₂). If these metabolites are oxidized to the electrophilic CE quinones (CE-Q), they may react with DNA. Specifically, the carcinogenic 4-CE (51, 76) are oxidized to CE-3,4-Q, which react with DNA to form depurinating adducts (50, 77). These adducts generate apurinic sites that may lead to oncogenic mutations (75, 77-79), thereby initiating cancer.

The breast is an endocrine organ and can synthesize E₂ *in situ* from precursor androgens via the enzyme aromatase (1). Breast tissue contains aromatase and produces amounts of E₂ that exert biologic effects on proliferation. The effects of local production exceed those exerted in a classical endocrine fashion by uptake of E₂ from plasma. One critical factor is excessive synthesis of E₂ by overexpression of CYP19 in target tissues (80-84) and/or the presence of excess sulfatase that converts stored E₁ sulfate to E₁ (85). The observation that breast tissue can synthesize E₂ *in situ* suggests that much more E₂ is present in some locations of target tissues than would be predicted from plasma concentration (84). A second critical factor might be high levels of 4-CE due to overexpression of CYP1B1, which converts E₂ predominantly to 4-OHE₂ (86-88). This could result in relatively large amounts of 4-CE and, subsequently, more extensive oxidation to their CE-3, 4-Q. A third factor could be a lack or low level of COMT activity. If this enzyme is insufficient, either through a low level of expression or its low activity allele, 4-CE will not be effectively methylated, but will be oxidized to the ultimate carcinogenic metabolite, CE-3, 4-Q. Fourth, a low level of GSH and/or low levels of quinone reductase and/or CYP reductase can leave available a higher level of CE-Q that may react with DNA.

The effects of some of these factors have already been observed in analyses of breast tissue samples from women with and without breast cancer (89). The levels of E₁ (E₂) in women with carcinoma were higher. In women without breast cancer, a larger amount of 2-CE than 4-CE was observed. In women with breast carcinoma, the 4-CE were 3.5 times more abundant than the 2-CE and were 4 times higher than in the women without breast cancer. Furthermore, a statistically lower level of methylation was observed for 2-CE and 4-CE in cancer cases *vs* controls. Finally, the level of CE-Q conjugates in women with cancer was 3 times that in the controls, suggesting a larger probability for the CE-Q to react with DNA in the breast tissue of women with carcinoma. The levels of E₁(E₂) ($p<0.02$) and quinone conjugates ($p<0.01$) are highly significant predictors of breast cancer, and the levels of methylated CE ($p<0.02$) are significant predictors of protection against breast cancer. Altogether, these data are supporting the concept that estrogen and its metabolites can be found at high concentration in the breast tissue indicating a direct carcinogenic effect in the breast epithelial cells (89).

A-iii- Estrogens as inducers of aneuploidy.

Breast cancer is considered the result of sequential changes that accumulate over time. DNA content changes, i.e., loss of heterozygosity (LOH) and aneuploidy, can be detected at early stages of morphological atypia, supporting the hypothesis that aneuploidy is a critical event driving neoplastic development and progression (90, 91). Aneuploidy is defined as the gain or loss of chromosomes; it is a dynamic, progressive, and accumulative event that is almost universal in solid tumors (92, 93). The extensive array of altered gene expression observed in tumors and the numerous altered chromosomes detected by CGH (19, 94) provide striking evidence that aneuploidy can totally disrupt cell homeostatic control. The main question is whether aneuploidy is a consequence of neoplastic development or a cause of neoplastic development (19, 20, 94). One of the several mechanisms proposed for the development of aneuploidy is the failure to appropriately segregate chromosomes (20, 21, 95). For example interference with mitotic spindle dynamics, abnormal centrosome duplication, altered chromosome condensation and

cohesion, defective centromeres, and loss of mitotic checkpoints (95). Functional consequences of centrosome defects may play a role during neoplastic transformation and tumor progression, increasing the incidence of multipolar mitoses that lead to chromosomal segregation abnormalities and aneuploidy. In considering estrogen as a carcinogenic agents there is evidence that they affect microtubules (96) and a recently report indicates that progesterone may facilitate aneuploidy (97). The importance of these findings is magnified with the recent publications that demonstrate women on hormone replacement treatments that include progesterone have increased mammographic breast density and increased breast cancer risk than women taking only estrogen (98-100).

In the center stage of the research endeavor on aneuploidy are the centrosomes that are organelles that nucleate microtubule growth and organize the mitotic spindle for segregating chromosomes into daughter cells, establishing cell shape and cell polarity, processes essential for epithelial gland organization (19,95). Centrosomes also coordinate numerous intracellular activities, in part by providing a site enriched for regulatory molecules, including those that control cell cycle progression, centrosome and spindle function, and cell cycle checkpoints (20, 101). Although the underlying mechanisms for the formation of abnormal centrosomes are not clear, several possibilities have been proposed and implicated in the development of cancer such as alterations of checkpoint controls initiating multiple rounds of centrosome replication within a single cell cycle and failure of cytokinesis, cell fusion, and cell cycle arrest in S-phase uncoupling DNA replication from centrosome duplication (102).

To fully demonstrate that estrogens are carcinogenic in the human breast through one or more of the mechanisms explained above it will require an experimental system in which, estrogens by itself or one of the metabolites would induce transformation phenotypes indicative of neoplasia in HBEC *in vitro* and also induce genomic alterations similar to those observed in spontaneous malignancies, such as DNA amplification and loss of genetic material that may represent tumor suppressor genes (103-118).

B-BODY

B-i-The *in vitro* model of cell transformation

The transforming potential of estrogens on human breast epithelial cells (HBEC) *in vitro*, have been evaluated by utilizing the spontaneously immortalized HBEC MCF-10F (119,120). The spontaneously immortalized MCF-10F cells, treated cells and derived clones were maintained in DMEM:F-12 [1:1] medium with a 1.05 mM Ca²⁺ concentration. All cell lines were regularly tested for correct identity using a fingerprint cocktail of three minisatellite plasmid probes (ATCC, Rockville, MD). Culture media were prepared by the Central Center Tissue Culture Facility at the Fox Chase Cancer (Philadelphia, PA). In order to mimic the intermittent exposure of HBEC to endogenous estrogens, all cells were first treated with 0, 0.007nM, 70nM and 1μM of E₂, DES, BP, Progesterone, 2-OH-E2, 4-OH-E2 and 16-α-OH E2 at 72 hrs and 120 hours post plating. Treatments were repeated during the second week, and cells were collected at the 14th day for phenotypic and genotypic analysis. At the end of each treatment period, the culture medium was replaced with fresh medium. At the end of the second week of treatment, the cells were assayed for determination of, survival efficiency (SE), colony efficiency (CE), colony size (CS), ductulogenic capacity and invasiveness in a reconstituted basement membrane [21, 22].

B-ii-Transformation effect of estrogens and its metabolites in MCF-10F cells

We have determined the optimal doses for the expression of the cell transformation phenotype by treating the immortalized human breast epithelial cells (HBEC) MCF-10F with 17 β -estradiol (E₂) with 0.0, 0.07 nM, 70 nM, or 1 μ M of E₂ twice a week for two weeks. The survival efficiency (SE) was increased with 0.007nM and 70 nM of 17 β estradiol and decrease with 1 μ M and the proliferative activity of these E2 transformed cells, measured by the percentage of cells in the S phase of the cell cycle, was also increased in a dose dependent fashion. The cells treated with either doses of E2 formed colonies in agar methocel and the size was not different among them, however, the CE increased from 0 in controls to 6.1, 9.2, and 8.7 with increasing E₂ doses.

Ductulogenesis was quantitatively evaluated by estimating the ability of the cell plated in collagen to form tubules or spherical masses (SM). Non-transformed cells produce ductules like structure and transformed cells produce spherical or solid masses of cells. Cells treated with DMSO, cholesterol or progesterone at different concentrations was unable to alter the ductular pattern. E2, BP and DES treated cells induces the loss of MCF10F cells to produce ductules in a dose dependent fashion and the number of solid masses paralleled the formation of colonies in agar methocel. Histological analysis shows that MCF10-F cells form ductules in collagen matrix that are lined by a single layer of cuboidal epithelial cells, this pattern was not disturbed by cholesterol or progesterone treatment. Most of the cells growing in the collagen matrix are actively proliferating as detected by immunostaining with Ki67.

2-OH-E₂, 4-OH-E₂, and 16 α -OH-E₂ induce the formation of colonies in agar methocel. Cells treated with cholesterol were unable to produce colonies. The size of the colonies was significantly smaller in those cells treated with 2-OH-E2 or progesterone. Whereas the number of colonies was dose dependent reaching its maximum efficiency at the concentration of 70nM for most of the compounds, 4-OH-E2 was the most efficient in inducing larger colonies and number at a doses of 0.007nM. E2, and BP behave very similar and are more transforming agents than DES and 2-OH-E2.

The metabolites of estrogen significantly impair the formation of ductules replacing them by structures filled by large cuboidal cells. Some of the cells present cytoplasmic vacuolization and piknosis. Cells treated with 2-OH E2 or 16- α -OH-E2 is less efficient in altering the ductulogenic capacity. Importantly 4-OH-E2 at a dose of 0.007nM induces significant changes in the ductulogenic capacity with a maximal number of solid masses. These structures also have a high proliferative index.

The invasiveness capacity of E2, DES, 4OH-E2 and BP transformed cells measured in the Boyden Chamber, was very high when compared with the control or those treated with DMSO, P, or 2OH-E2.

B-iii-Antiestrogens in the expression of the transformation phenotype

The proliferative activity of the MCF-10F cells that has been treated with Tamoxifen alone or ICI-182,780 was not modified when compared with the control. Instead those cells that were treated with 17- β -estradiol in presence of Tamoxifen or ICI-182,780 showed no increment of the proliferative activity neither in monolayer nor collagen matrix. The colony formation in agar methocel was abrogated and the ductulogenic capacity was maintained. The proliferative activity of these cells in collagen matrix was also abrogated. 4-OH-E2 transforming efficiency was not abrogated by ICI neither in the colony efficiency assay nor in the loss of ductulogenic capacity. The histology of the solid masses induced by 4-OH estradiol in collagen matrix were not modified by ICI, even the number of cells was significantly

higher. ICI-182,780 was unable to abrogate the invasive phenotype induced by estrogen and tamoxifen even exacerbate the invasive phenotype.

B-IV-Detection of estrogen receptors in MCF 10F cells

The ER alpha was not detected in the MCF-10F cells or in those transformed by estrogens or its metabolites. The positive control MCF-7 cells was positive for ER alpha showing by Western blot the specific band corresponding to a 67 kDa, instead the band was absent in the negative control MDA-MB-235 cell line. The ER beta protein expression analysis showed two bands 68 and 53 kDa of molecular weight corresponding to ER beta long and short form, respectively. Both bands were present in the MCF-10F cells and in the transformed cells. Those cells transformed by 17 β estradiol as well as those treated with progesterone significantly overexpressed the long form of ER beta. Instead, MCF-7 cells showed the short form of the ER beta.

The progesterone receptor (PR) expression was negative in the MCF-10F cells when compared with MCF-7 cells that was used a positive control presenting the 186 and 82 kDa PR long and short form respectively. The estrogen-transformed cells also expressed PR.

B-v-Genomic changes induced by estrogen and its metabolites in the transformation of human breast epithelial cells.

In order to determine if the gene expression profile induced by E2, 4-OH estradiol and BP were the same or whether they are divergent in their pattern of expression, mRNA from these transformed cells was extracted and hybridized to cDNA array membranes that contained 1,176 human genes (Clontech Human Cancer 1,2 array). The genomic signature of the three transformed cells present a cluster of genes that are commonly unregulated (Table 1), indicating that a similar mechanism is involved in the transformation pathway. Interestingly there are genes that are upregulated in the E₂ and 4-OH-E₂ transformed cells such as the CENP-E (Table 2) that are not modified in the BP transformed cells. The same occurs for several genes that are downregulated differentially in the three transformed cells (Table 3).

Table 1
Common up-regulated genes in MCF-10F cells transformed by Bp, E2 and 4OH using cDNA array

Gene Description	Swissprot #	Function	Bp/10F	E2/10F	4OH/10F
c-myc oncogene	P01106	Oncogene	3.24	3.66	6.21
fos-related antigen	P15407	Oncogene	10.25	2.31	15.04
HER3	P21860	Oncogene	2.09	3.32	7.95
SRF accessory protein 2	P41970	Transcription	3.61	2.46	9.11
hEGR1	P18146	Transcription	3.2	6.49	2.91
Splicing factor 9G8	Q16629	mRNA processing	2.23	2.93	4.42
antigen KI-67	P46013	Cell proliferation	3.2	2.7	5.97
HMG-I	P17096	Chromatin	2.36	3.26	7.95
nm23-H4	O00746	Kinase	2.02	2	2.24
cytokeratin 2E	P35908	Keratin	43.09	2.38	4.37

Table 2

Common down-regulated genes in MCF-10F cells transformed by Bp, E2 and 4OH using cDNA array

Array Location	Gene Description	Swissprot #	Function	Bp/10F	E2/10 F	4OH/10 F
A11g	PIG7	Q99732	Tumor suppressor	0.02	0.04	0.19
A14h	CD82 antigen	P27701	Tumor suppressor	0	0.18	0
B06k	rho GDP dissociation inhibitor 2	P52566	Tumor suppressor	0	0	0.21
A02g	neurogenic locus notch protein	Q04721	Transcription	0.29	0.47	0.38
A13h	active breakpoint cluster region-related protein	Q12979	Transcription	0.13	0.25	0.46
A14c	ets-related protein tel	P41212	Transcription	0	0.08	0.08
C06m	B4-2 protein	Q12796	Transcription	0	0	0
B03n	T3 receptor-associating cofactor 1	O00613	Intracellular transducers	0.48	0.41	0.22
E04b	HDGF	P51858	Growth factor	0.34	0.1	0.24
F07i	HNRNPK	Q07244	mRNA processing	0	0	0.17
B02j	RalB GTP-binding protein	P11234	G protein	0	0.24	0
B04j	rhoC	P08134	G protein	0.09	0.06	0.48
B12j	p21-rac2	P15153	G protein	0.12	0.2	0.49
B13i	p21-rac1	P15154	G protein	0	0	0.33
A06j	CDK5	Q00535	Kinase	0.18	0	0.41
B05h	NDR protein kinase	Q15208	Kinase	0	0	0
B08c	tissue-specific extinguisher 1	P10644	Kinase	0	0	0.19
A09l	CDKN1A	P38936	Kinase inhibitor	0.09	0.03	0.08
A10d	HGF-SF receptor	P08581	Kinase inhibitor	0	0	0.31
B02m	hint protein	P49773	Kinase inhibitor	0	0	0.37
B07l	calvasculin	P26447	Calcium-binding	0	0.11	0.46
B09n	CD27 ligand	P32970	Death receptor ligand	0.37	0	0
C02c	BAG-1	Q99933	BCL family protein	0	0	0.19
C09m	AH receptor	P35869	Nuclear receptor	0.06	0.12	0
F04i	lipocalin 2	P80188	Trafficking	0	0	0
F09h	TRAM protein	Q15629	Trafficking	0	0	0.29
F10h	dual-specificity A-kinase anchoring protein 1	Q92667	Targeting	0	0.19	0.24
D01d	cadherin 3)	P22223	Cell adhesion	0.32	0.14	0.08
D02e	integrin beta 6 precursor	P18564	Cell adhesion	0.16	0.11	0.22
E02f	IGF-binding protein 3	P17936	Hormone	0	0	0
E02m	HLA-C	Q30182	Immune	0.19	0.17	0
E02n	GRP 78	P11021	Immune	0	0	0
F03b	fibronectin precursor	P02751	Extracellular matrix	0.32	0.13	0.09
F13n	insulin-induced protein 1	O15503	Unclassified	0.13	0.33	0.35
F08m	PM5 protein	Q15155	Unclassified	0.17	0.34	0

Table 3**Specific up-regulated genes in BP-transformed cells by cDNA array**

Array Location	Gene Description	Swissprot #	Function	BP/10F
C051	RAR-gamma 1	P13631	Transcription	3.77
B04k	caveolin-1	Q03135	Signaling	3.35
A03b	ezrin	P15311	Oncogene	2.01
C04h	HHR23A	P54725	Stress response	2.04
C08g	mutL protein homolog	P40692	Stress response	4.31
E07h	glycosylation-inhibiting factor	P14174	Cell communication	4.44
D06e	integrin beta 4	P16144	Cell adhesion	4.24
D08e	integrin alpha 7B precursor	Q13683	Cell adhesion	3.06
D05e	integrin alpha 6 precursor	P23229	Cell adhesion	2.24
D07e	integrin alpha 1	P56199	Cell adhesion	2.31
F05d	LDHA	P00338	Carbohydrate metabolism	6.25
F08f	cytokeratin 18	P05783	Cytokeratin	3.04
F14e	BIGH3	Q15582	Microfilament	6.73

B-vi-Chromosomal alterations induced by estrogens and its metabolites.

During the process of cell transformation induced by estrogen and its metabolites there is an increase in the number of multinucleated cells and abnormal mitoses that is associated with the overexpression of one component of the centromere-kinetochore complex CENP-E. It is important to emphasize that the percentage of these abnormal mitoses is less than 1%. The movements that chromosomes undergo during mitosis are facilitated by the mitotic spindle, an apparatus composed principally of microtubule fibers that attach to a pair of kinetochores located on opposite sides of the centromere region of chromosomes. The microtubule-kinetochore interaction is essential for chromosome segregation. Disruptions of this interaction will lead to unequal distribution of chromosomes in daughter cells (123). We have found that the CENP-E, a *ca.* 300 kDa protein that have been recently identified to be a novel member of the kinesin superfamily of microtubule-based motor proteins (123) is overexpressed in MCF-10F transformed cells by estrogens and its metabolites but not in the BP transformed cells. CENP-E staining appeared only in mitotic cells (123), suggesting that it is a mitosis-specific motor. Its association with kinetochores suggests that it functions to translocate chromosomes along the spindle microtubules. This phenomena, however, was not observed in the BP transformed cells indicating that whereas aneuploidy is part of the neoplastic transformation process is depending of the carcinogenic insult and probably not the main driving force to cause genomic instability. This concept was further confirmed by the lack of significant karyotypic changes detected in these transformed cells and by the fact that the same cluster of genes were overexpressed in cells transformed with E2, 4-OH-E2 and BP (Table 1), indicating that there is a common pathway of transformation and that may be responsible for driving the normal cell to neoplasia. The data also point toward the concept that certain compounds like steroid hormones or its metabolites may affect certain genes more readily than other exerting the expression of genes that are altering the mitotic spindle

and therefore making the cell aneuploid. However, does not support the concept that aneuploidy is the driving force of transformation but a consequence of it.

B-vii-LOH in HBEC treated with estrogen and its metabolites.

Genomic DNA was analyzed for the detection of micro-satellite DNA polymorphism using 64 markers covering chromosomes (chr) 3, 11, 13 and 17. We have detected loss of heterozygosity (LOH) in ch13q12.2-12.3 (D13S893) and in ch17q21.1 (D17S800) in E₂, 2-OH-E₂, 4-OH-E₂-, E₂+ICI, E₂+Tamoxifen and BP treated cells. LOH in ch17q21.1-21.2 (D17S806) was also observed in E₂, 4-OH-E₂, E₂+ICI, E₂+Tamoxifen and BP-treated cells. MCF-10F cells treated with P or P+E₂ did not show LOH in the any of the markers studied. LOH was strongly associated with the invasion phenotype. Altogether our data indicate that E₂ and its metabolites induce in HBEC LOH in loci of chromosomes 13 and 17, that has been reported in primary breast cancer, that the changes are similar to those induced by the chemical carcinogen (BP) and that the genomic changes were not abrogated by antiestrogens.

C-KEY RESEARCH ACCOMPLISHMENTS

C-i-Short term treatment of HBEC with physiological doses of 17- β estradiol induces anchorage independent growth, colony formation in agar methocel, and reduced ductulogenic capacity in collagen gel, all phenotypes whose expression is indicative of neoplastic transformation, and that are induced by BP under the same culture conditions.

C-ii-Progesterone was unable to induce significant increase in colony formation, although small colonies less than 50 μ m in diameter were observed, whereas none were found in the MCF10F cells treated with DMSO or cholesterol. The ductulogenic pattern was not impaired by progesterone but the luminal size was smaller that those found in the MCF10F cells treated with DMSO or cholesterol.

C-iii-The fact that the MCF10F cells are ER α negative, indicate that this receptor pathway is not involved in the carcinogenic process. Although the presence of ER- β may indicate that the response of the cells to growth and form colonies in agar methocel could be mediated by this receptor. This is supported by the fact that either tamoxifen or a pure antiestrogen like ICI abrogated these phenotypes.

C-iv-The Invasion phenotype, an important marker of tumorigenesis is not modified when the cells are treated in presence of tamoxifen or ICI, suggesting that other pathways may be involved. Although we cannot rule out the possibility, that 4-OH-E₂ may interact with other receptors still not identified, with the data presently available the direct effect of 4-OH-E2 at so low doses support the concept that metabolic activation of estrogens mediated by various cytochrome P450 (CYP) complexes, generating through this pathway reactive intermediates that elicit direct genotoxic effects leading to transformation. An increase in catechol estrogen (4-OH-E2) due to either elevated rates of synthesis or reduced rates of monomethylation will easily lead to their autoxidation to semiquinones and subsequently quinones, both of which are electrophiles capable of covalently binding to nucleophilic groups on DNA. Through this pathway estrogen metabolites exert direct genotoxic effects that might increase mutation rates, or compromise the DNA repair system, leading to the accumulation of genomic alterations essential to tumorigenesis. This assumption was confirmed when we found that all the transformation phenotypes induced by 4-OH-E2 were not abrogated when this compound was used in presence of the pure antiestrogenic ICI. The novelty of these observation lies in that the ER- β pathway in transformation

can successfully bypassed by the estrogen metabolite 4-OH E2.

C-v- Genomic DNA was analyzed for the detection of micro-satellite DNA polymorphism using 64 markers covering chromosomes (chr) 3, 11, 13 and 17. We have detected loss of heterozygosity (LOH) in ch13q12.2-12.3 (D13S893) and in ch17q21.1 (D17S800) in E₂, 2-OH-E₂, 4-OH-E₂-, E₂+ICI, E₂+Tamoxifen and BP treated cells. LOH in ch17q21.1-21.2 (D17S806) was also observed in E₂, 4-OH-E₂, E₂+ICI, E₂+Tamoxifen and BP-treated cells. MCF-10F cells treated with P or P+E₂ did not show LOH in the any of the markers studied.

C-vi-LOH was strongly associated with the invasion phenotype. Altogether our data indicate that E₂ and its metabolites induce in HBEC LOH in loci of chromosomes 13 and 17, that has been reported in primary breast cancer, that the changes are similar to those induced by the chemical carcinogen (BP) and that the genomic changes were not abrogated by antiestrogens.

D-REPORTABLE OUTCOMES

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E-CONCLUSIONS

In the present work we demonstrate that estradiol and its metabolites mainly 4-OH estradiol are able to induce transformation phenotypes in the human breast epithelial cells (HBEC) MCF-10F. The fact that the MCF10F cells are ER α negative, indicate that this receptor pathway is not involved in the carcinogenic process. Although the presence of ER- β may indicate that the response of the cells to growth and form colonies in agar methocel could be mediated by this receptor, the Invasion phenotype, an important marker of tumorigenesis, is not modified when the cells are treated in presence of tamoxifen or ICI. We cannot rule out the possibility, that 4-OH-E₂ may interact with other receptors still not identified, with the data presently available the direct effect of 4-OH-E2 at so low doses support the concept that metabolic activation of estrogens mediated by various cytochrome P450 (CYP) complexes, generating through this pathway reactive intermediates that elicit direct genotoxic effects leading to transformation. An increase in catechol estrogen (4-OH-E2) due to either elevated rates of synthesis or reduced rates of monomethylation will easily lead to their autoxidation to semiquinones and subsequently quinones, both of which are electrophiles capable of covalently binding to nucleophilic groups on DNA. Through this pathway estrogen metabolites exert direct genotoxic effects that might increase mutation rates, or compromise the DNA repair system, leading to the accumulation of genomic alterations essential to tumorigenesis. This assumption was confirmed when we found that all the transformation phenotypes induced by 4-OH-E2 were not abrogated when this compound was used in presence of the pure antiestrogenic ICI. We have detected loss of heterozygosity (LOH) in ch13q12.2-12.3 (D13S893) and in ch17q21.1 (D17S800) in E₂, 2-OH-E₂, 4-OH-E₂-, E₂+ICI, E₂+Tamoxifen and BP treated cells. LOH in ch17q21.1-21.2 (D17S806) was also observed in E₂, 4-OH-E₂, E₂+ICI, E₂+Tamoxifen and BP-treated cells. MCF-10F cells treated with P or P+E₂ did not show LOH in the any of the markers studied. LOH was strongly associated with the invasion phenotype. Altogether our data indicate that E₂ and its metabolites induce in HBEC LOH in loci of chromosomes 13 and 17, that has been reported in primary breast cancer, that the changes are similar to those induced by the chemical carcinogen (BP) and that the genomic changes were not abrogated by antiestrogens.

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APPENDIX

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Estrogens and Breast Cancer

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Table of Contents

Publications:

- 1.-Russo, J., Hu, Y.F., Tahin, Q., Mihaila, D., Slater, C., Lareef, M.H. and Russo, I.H. Carcinogenicity of Estrogens in Human breast epithelial cells. *Acta Pathologica, Microbiologica Immunologica Scandinavica (APMIS)* 109:39-52, 2001.
- 2.-Russo, J., Lareef, M.H., Tahin, Q., Hu, Y-F., Slater, C.M., Ao, X., and Russo, I.H. 17 beta estradiol is carcinogenic in human breast epithelial cells. *Journal of Steroid Biochemistry and Molecular Biology* 80(2):149-162, 2002.
- 3.-Russo, J. Tahin, Q., Lareef, H.M., Hu, YF. Russo, I.H. Neoplastic transformation of human breast epithelial cells by estrogens and chemical carcinogens. *Environmental and Molecular Mutagenesis.* 39(2): 254-263, 2002.



17 β -Estradiol is carcinogenic in human breast epithelial cells

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Abstract

The association found between breast cancer development and prolonged exposure to estrogen suggests that this hormone is of etiologic importance in the causation of this disease. In order to prove this postulate, we treated the immortalized human breast epithelial cells (HBEC) MCF-10F with 17 β -estradiol (E₂) for testing whether they express colony formation in agar methocel, or colony efficiency (CE), and loss of ductulogenesis in collagen matrix, phenotypes also induced by the carcinogen benz[a]pyrene (BP). MCF-10F cells were treated with 0.0, 0.007, 70 nM, or 0.25 mM of E₂ twice a week for 2 weeks. CE increased from 0 in controls to 6.1, 9.2, and 8.7 with increasing E₂ doses. Ductulogenesis was 75 \pm 4.9 in control cells; it decreased to 63.7 \pm 28.8, 41.3 \pm 12.4, and 17.8 \pm 5.0 in E₂-treated cells, which also formed solid masses or spherical formations lined by a multilayer epithelium, whose numbers increased from 0 in controls to 18.5 \pm 6.7, 107 \pm 11.8 and 130 \pm 10.0 for each E₂ dose. MCF-10F cells were also treated with 3.7 μ M of progesterone (P) and the CE was 3.39 \pm 4.05. At difference of E₂, P does not impaired the ductulogenic capacity. Genomic analysis revealed that E₂-treated cells exhibited loss of heterozygosity in chromosome 11, as detected using the markers D11S29 and D11S912 mapped to 11q23.3 and 11q24.2–25, respectively. These results also indicate that E₂, like the chemical carcinogen BP, induces in HBEC phenotypes indicative of neoplastic transformation. © 2002 Elsevier Science Ltd. All rights reserved.

Keywords: 17 β -Estradiol; HBEC; Estrogen; Breast

1. Introduction

Epidemiological and clinical evidence indicate that breast cancer risk is associated with prolonged exposure to female ovarian hormones [1–4]. Breast cancer is a hormone- and sex-dependent malignancy whose development is influenced by a myriad of hormones and growth factors [5,6], from which estrogens have been demonstrated to be of essential importance in this phenomenon as it is observed in postmenopausal hyperestrogenism resulting from the use of estrogenic hormone replacement therapy and obesity [7,8].

Estrogens, that are necessary for the normal development of both reproductive and non-reproductive organs, exert their physiological effects by binding to their specific receptors, the estrogen receptors (ER)- α or - β [9–16]. Estrogens might act as well through alternate non-receptor mediated pathways [17]. E₂, under the effect of 17 β -oxidoreductase is continuously interconverted to estrone (E₁), and both are hydroxylated at C-2, C-4, or C-16 α positions by cytochrome P450 isoenzymes, i.e. CYP1A1, CYP1A2, or CYP1B1, to

form catechol estrogens [18–23]. The demonstration that the catecholestrogen 4-hydroxyestradiol (4-OH-E₂) induces an estrogenic response in the uterus of ER- α null mice, and the fact that this response is not inhibited by the antiestrogen ICI-182, 780 [9], indicate that this catecholestrogen does not exert its effect on the ER. The metabolic activation of estrogens can be mediated by various cytochrome P450 (CYP) complexes, generating through this pathway reactive intermediates that elicit direct genotoxic effects by increasing mutation rates. An increase in CE due to either elevated rates of synthesis or reduced rates of monomethylation will easily lead to their autoxidation to semiquinones and subsequently quinones, both of which are electrophiles capable of covalently binding to nucleophilic groups on DNA via a Michael addition and thus, serve as the ultimate carcinogenic reactive intermediates in the peroxidative activation of CE. Thus, estrogen and estrogen metabolites exert direct genotoxic effects that might increase mutation rates, or compromise the DNA repair system, leading to the accumulation of genomic alterations essential to tumorigenesis [18–23]. Although this pathway has been demonstrated in other systems [18–20], it still needs to be demonstrated in human breast epithelial cells (HBECs).

Furthermore, if estrogen is carcinogenic in the human breast through the above-mentioned pathway, it would induce

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in breast epithelial cells in vitro transformation phenotypes indicative of neoplasia and also induce genomic alterations similar to those observed in spontaneous malignancies, such as DNA amplification and loss of genetic material that may represent tumor suppressor genes [24–39]. In order to test this hypothesis, we have evaluated the transforming potential of E₂ on HBEC in vitro, utilizing the spontaneously immortalized HBEC MCF-10F [40,41]. This cell line lacks both ER- α and ER- β , although this latter receptor is induced in cells transformed by chemical carcinogens [42]. In the present work, we report that the same phenotypes and characteristics that were expressed by MCF-10F cells transformed by the chemical carcinogen benz[a]pyrene (BP) and oncogenes [43–46] were expressed in E₂-treated cells. E₂ transformed cells exhibited loss of heterozygosity (LOH) in loci of chromosome 11, known to be affected in spontaneously occurring breast lesions, such as ductal hyperplasia, carcinoma in situ, and invasive carcinoma [47–60].

2. Material and methods

2.1. Cells and dose selection

MCF-10F cells at passage 125 were cultured in DMEM:F-12 medium containing 1.05 mM calcium (Ca²⁺), antibiotics, antimycotics, hormones, growth factors, and equine serum as previously described [44]. In order to determine the optimal doses for the expression of the cell transformation phenotype, we treated the immortalized HBEC MCF-10F with 0.0, 0.07 nM, 70 nM, or 0.25 mM of 17 β -estradiol (E₂) twice a week for 2 weeks (Fig. 1). Based upon these results a dose of 3.7 μ M (1 μ g/ml) was selected for testing the effect of E₂, progesterone (P), and BP.

2.2. Evaluation of the effect of estrogens and other compounds on the expression of cell transformation phenotypes

The spontaneously immortalized MCF-10F cells, treated cells and derived clones were maintained in DMEM:F-12

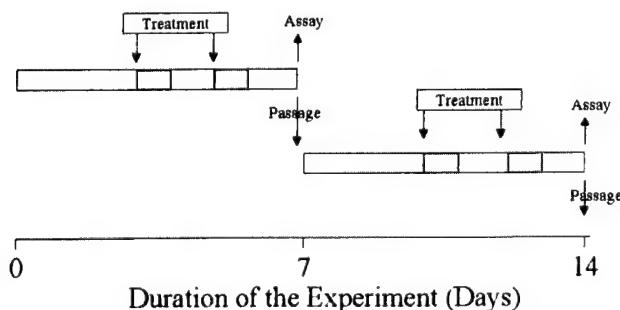


Fig. 1. MCF-10F cells were treated with E₂, DES, or BP at 72 and 120 h post plating. Treatments were repeated during the second week, and cells were collected at the 14th day for phenotypic and genotypic analysis.

(1:1) medium with a 1.05 mM Ca²⁺ concentration. All cell lines were tested for correct identity using a fingerprint cocktail of three minisatellite plasmid probes (ATCC, Rockville, MD). Culture media were prepared by the Central Center Tissue Culture Facility at the Fox Chase Cancer (Philadelphia, PA). MCF-10F cells were treated with 1.0 μ g/ml E₂ (Aldrich, St. Louis, MO), progesterone (Sigma Chemical Co., St. Louis, MO), control cells were treated with DMSO. MCF-10F cells treated with 1.0 μ g/ml BP served as positive controls for cell transformation assays. In order to mimic the intermittent exposure of HBEC to endogenous estrogens, all cells were first treated with E₂, P, or BP at 72 and 120 h post-plating. At the end of the first week of treatment, the cells were passaged for administration of another two periods of hormonal treatment. Treatments were repeated during the second week, and cells were collected at the 14th day for phenotypic and genotypic analysis (Fig. 1). At the end of each treatment period, the culture medium was replaced with fresh medium. At the end of the second week of treatment the cells assayed for determination of survival efficiency (SE), colony efficiency (CE), colony size (CS), and ductulogenic capacity, as described in previous publications [44,45].

2.3. Colony formation in agar-methocel assay

This technique was utilized as an in vitro assay for anchorage independent growth, a parameter indicative of transformation. Parental, control, and treated cells were suspended at a density of 2×10^4 cells/ml in 2 ml of 0.8% methocel (Sigma Chemical Co., St. Louis, MO) dissolved in DMEM:F-12 (1:1) medium containing 20% horse serum. Cells from each treatment group and time point were plated in four 24-well chambers pre-coated with 0.5 ml of 0.8% agar base in DMEM:F-12 medium, which was replaced with fresh feeding medium containing 0.8% methocel twice a week. The actual number of cells plated was calculated as the average of cells counted at 10 \times magnification in five individual fields, and multiplied by a factor of 83. CE and CS were measured 21 days after plating. CE was determined by a count of the number of colonies greater than 50 μ m in diameter, and expressed as a percentage of the original number of cells plated per well.

2.4. Ductulogenesis in collagen matrix

This in vitro technique evaluates the capacity of cells to differentiate by providing evidence of whether treated cells form three-dimensional structures when grown in a collagen matrix. Parental, control, and treated cells were suspended at a final density of 2×10^3 cells/ml in 89.3% Nitrogen¹⁰⁰ collagen matrix (Collagen Co., Palo Alto, CA) and plated into four 24 well chambers pre-coated with agar base. The cells were fed fresh feeding medium containing 20% horse serum twice a week. The cells were examined under an inverted microscope for a period of 21 days or longer for determin-

ing whether they formed ductule-like structures or whether they grew as unorganized clumps. The final structures were photographed, and then fixed in 10% neutral buffered formalin, embedded in paraffin, sectioned, and stained with hematoxylin–eosin for histological examination.

2.5. Genomic analysis of treated cells

2.5.1. DNA isolation

To obtain DNA, treated and control cells were lysed in 5 ml of TNE (0.5 M Tris, pH 8.9, 10 mM NaCl, 15 mM EDTA) with 500 µg/ml proteinase K and 1% sodium dodecyl sulfate (SDS), and incubated at 48 °C for 24 h. Following two extractions with phenol (equilibrated with

0.1 M Tris, pH 8.0), the DNA was spooled from 2% v/v of 100% ethanol, air dried and resuspended in 20 mM EDTA. The DNA was then treated sequentially with RNase A (100 µg/ml) for 1 h at 37 °C and 100 µg/ml proteinase K, 1% SDS, at 48 °C for 3 h, followed by two extractions with saturated phenol. The DNA was again retrieved from the aqueous phase by ethanol precipitation, washed extensively in 70% ethanol, and after air-drying suspended in TE (10 mM Tris, pH 8.0), 1 mM EDTA.

2.5.2. Detection of allelic loss

We evaluated for allelic losses the regions of chromosomes 1, 2, 3, 6, 8, 9, 11, 12, 13, 16, 17, and 18 most frequently reported to exhibit LOH in spontaneous breast

Table 1
Microsatellite DNA polymorphism analysis of MCF-10F cells treated with E₂ or BP

Chromosome	Marker	Location	MCF10-F	E ₂ -1	E ₂ -2	BP
1	D1S104	1p21–1p23	○	○	○	○
1	BAT-40	1p13.1	○	○	○	○
2	D2S171	2p24–21	○	○	○	○
2	D2S123	2p16	○	○	○	○
3	D3S1297		○	○	○	○
3	D3S1560	3p26–3p25	○	○	○	○
3	D3S1304	3p26–3p25	○	○	○	○
3	D3S1307	3p26–p25	○	○	○	○
3	D3S1289	3p23–3p21	○	○	○	○
3	D3S1449	3p22.3–3p21.3	○	○	○	○
3	D3S1478	3p2103–21.2	○	○	○	○
3	D3S2384	3p21.3–21.2	○	○	○	○
3	D3S1450	3p21.1–3p14.2	○	○	○	○
3	D3S1217	3p21	○	○	○	○
3	D3S1447	3p21	○	○	○	○
3	D3S1241	3p21	○	○	○	○
3	D3S1448	3p21	○	○	○	○
3	D3S1480	3p14	○	○	○	○
6	ESR	6q24–27	○	○	○	○
8	MYCL-1	8q24.1	○	○	○	○
9	D9S199	9p23	○	○	○	○
9	D9S157	9p23–22	○	○	○	○
9	D9S171	9p21	○	○	○	○
9	D9S165	9p21	○	○	○	○
11	D11S988	11pter–qter	○	○	○	○
11	D11S922	11p15.5	○	○	○	○
11	H-RAS1	11p15.5	○	○	○	○
11	CCKBR	11p15.4	○	○	○	○
11	D11S1392	11p13	○	○	○	○
11	Int-2	11p13.3	○	○	○	○
11	D11S907	11p13	○	○	○	○
11	D11S911	11q13–11p23	○	○	○	○
11	D11S436	11p12–11p11.1	○	○	○	○
11	D11S614	11q22–11q23	○	○	○	○
11	D11S940	11q22	○	○	○	○
11	DRD2	11q23.1	○	○	○	○
11	D11S968	11q23.01–11q25	○	○	○	○
11	D11S29	11q23.3	○	●	●	○
11	D11S925	11q23.3–11q24	○	○	○	○
11	D11S912	11q24.2–11q25	○	●	●	○
12	IGF-1	12q22–12q23	○	○	○	○
13	D13S289	13q12.2	○	○	○	○
13	D13S260	13q12.3	○	○	○	○

Table 1 (Continued)

Chromosome	Marker	Location	MCF10-F	E ₂ -1	E ₂ -2	BP
13	D13S267	13q2.3	○	○	○	○
13	D13S171	13q12.3–13	○	○	○	○
13	D13S218	13q13–14.1	○	○	○	○
13	GABRB-1	13q14.2	○	○	○	○
13	D13S155	13q14.3–21.2	○	○	○	○
16	D16S540		○	○	○	
17	D17S849	17p13.3	○	○	○	○
17	D17S796	17p13.1	○	○	○	○
17	D17S513	17p13.1	○	○	○	○
17	Tp53	17p13.1	○	○	○	○
17	D17S786	17p13.1	○	○	○	○
17	D17S793	17p13.1–7p11.2	○	○	○	○
17	D17S945	17p13–12	○	○	○	○
17	D17S520	17p12	○	○	○	○
17	D17S800	17q11.1–12	○	○	○	○
17	THRA-1	17q11.2–12	○	○	○	○
17	D17S787	17q21–22	○	○	○	
17	D17S855	17q21.2	○	○	○	○
17	D17S1323	17q21.2	○	○	○	○
17	D17S808	17q23.2	○	○	○	○
17	D17S789	17q24	○	○	○	○
17	D17S515	17q24.2–25.2	○	○	○	○
17	D17S785	17q25.2	○	○	○	○
18	D18S58	18q22.3–23	○	○	○	○

tumors (Table 1). DNA amplification of microsatellite length polymorphisms was utilized for detecting allelic losses present in the transformed clones. Microsatellites are polymorphic markers used primarily for gene mapping which can be broadly defined as relatively short (<100 bp) runs of tandem repeated di- to tetranucleotide sequence motifs [61–63]. The origin and nature of these polymorphism sequences is not well established, but they may result from errors of the polymerase during replication and/or from slightly unequal recombination between homologous chromatids during meiosis. These microsatellites have proven to be useful markers for investigating LOH and could be applicable to allelotyping as well as regional mapping of deletions in specific chromosomal regions. They are highly polymorphic, very common (between 10⁵ and 10⁶ per genome), and are flanked by unique sequences that can serve as primers for polymerase chain reaction (PCR) amplification [64].

2.5.3. DNA fingerprinting

Before performing DNA amplification of microsatellite DNA polymorphisms to detect allelic losses present in E₂-, DES-, and BP-treated cells, we verified by DNA fingerprinting whether all the clones derived from MCF-10F-treated cells were from the same lineage. Genomic DNA was extracted from the cells listed in Table 1. The identity of these cells was confirmed by Southern blot hybridization of genomic DNA with a cocktail of the three minisatellite probes D2S44, D14S13 and D17S74. Genomic DNAs were digested with *Hinf*I, and hybridized with probes under standard condition [64].

2.5.4. PCR analysis of microsatellites

Primers used for the analysis of microsatellite polymorphisms are given elsewhere [64]. Conditions for PCR amplification were as follows: 30 ng of genomic DNA, 100 pmol of each oligonucleotide primer, 1 × PCR buffer (Perkin-Elmer Cetus), 5 μM each of TTP, dCTP, dGTP, and dATP, 1 mCi [³²P] dATP (300 mCi/mmol) (DuPont, NEN, Boston, MA), and 0.5 units of AmpliTaq DNA polymerase (Perkin-Elmer Cetus) in 10 ml volumes. The reactions were processed through 27 cycles of 1 min at 94 °C, 1 min at the appropriate annealing temperatures determined for each set of primers, and 1 min at 72 °C; with a final extension of 7 min at 72 °C. Reaction products were diluted 1:2 in loading buffer (90% formamide, 10 mM EDTA, 0.3% bromophenol blue, and 0.3% xylene cyanol), heated at 90 °C for 5 min and loaded (4 ml) onto 5–6% denaturing polyacrylamide gels. After electrophoresis, gels were dried at 70 °C and exposed to XAR-5 film with a Lightning Plus intensifying screen at –80 °C for 12–24 h. Allele sizes were determined by comparison to M13mp18 sequencing ladders.

2.5.5. Detection of allelic loss

LOH was defined as a total loss of, or a 50%, or more reduction in density in one of the heterozygous alleles. All experiments were repeated at least three times to avoid false positive or false negative results. To control for possible DNA degradation, the same blots used to assess allelic loss were analyzed with additional DNA gene probes that detect large fragments. The bands were quantitated using a UltraScan XL laser densitometry (Pharmacia LKB Biotechnology Inc.) within the linear range of the film.

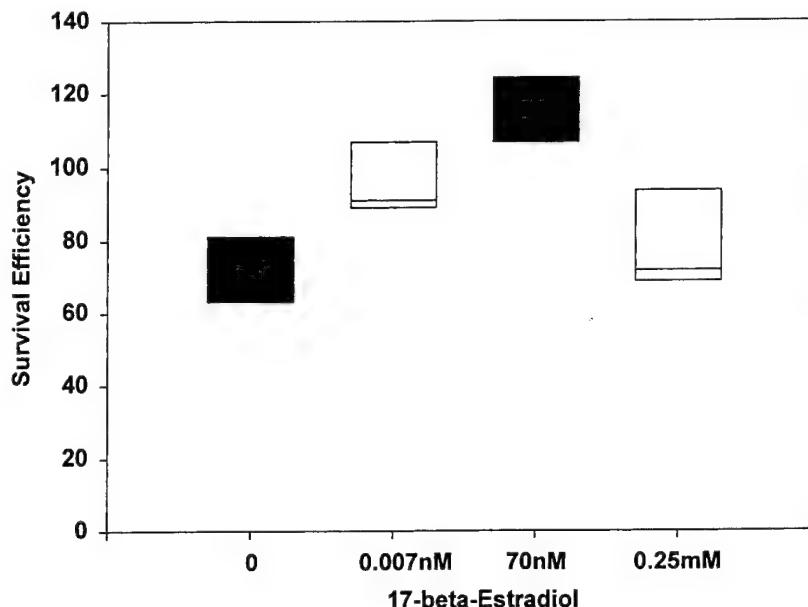


Fig. 2. Box plot showing the dose effect of 17 β -estradiol on the survival efficiency in agar methocel of MCF-10F cells.

3. Results

3.1. Determination of the dose response curve to 17 β -estradiol

In order to determine the optimal doses for the expression of the cell transformation phenotype we treated the immortalized HBEC MCF-10F with E₂ for testing the SE whether they express colony formation in agar methocel, or CE, and loss of ductulogenesis in collagen matrix. MCF-10F cells were treated with 0.0, 0.007, 70 nM, or 0.25 mM of E₂ twice

a week for 2 weeks. The SE was increased with 0.007 and 70 nM of 17 β -estradiol and decrease with 0.25 mM (Fig. 2). The cells treated with either doses of E₂ formed colonies in agar methocel and the size was not different among them (Fig. 3), however, the CE increased from 0 in controls to 6.1, 9.2, and 8.7 with increasing E₂ doses (Fig. 4). Ductulogenesis or the number of ductules per 10,000 cells plated, was 75 ± 4.9 in control cells; it decreased to 63.7 ± 28.8 , 41.3 ± 12.4 , and 17.8 ± 5.0 in E₂-treated cells (Fig. 5), which also formed spherical like structures or solid masses (Fig. 6a-d), whose numbers increased from 0 in controls to

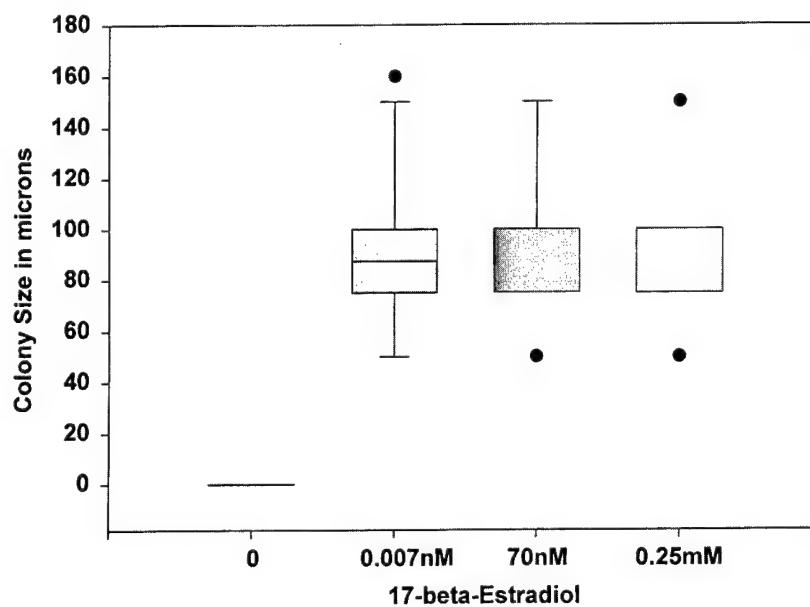


Fig. 3. Box plot showing the dose effect of 17 β -estradiol on colony size.

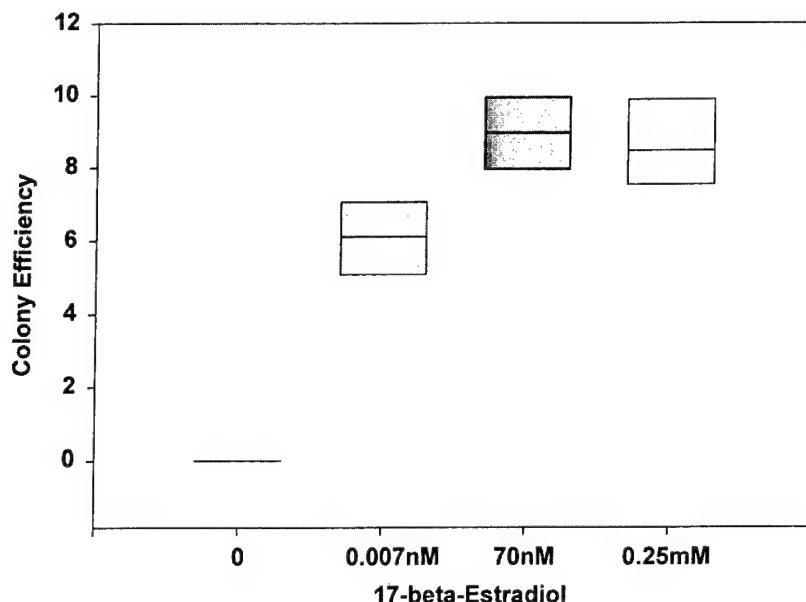


Fig. 4. Box plot showing the dose effect of 17 β -estradiol on colony efficiency.

18.5 \pm 6.7, 107 \pm 11.8 and 130 \pm 10.0 for each E₂ dose (Fig. 7).

3.2. Effect of estrogen, progesterone and benz[a]pyrene on the expression of transformation phenotypes

The SE of MCF-10F cells was increased with all the treatments (Fig. 8). Evaluation of colony formation at the end of the second week of E₂ and BP treatment revealed that MCF-10F cells formed colonies in agar-methocel over

60 μ m in diameter, whereas those cells treated with progesterone the colonies are smaller (Fig. 9). MCF-10F control cells treated with DMSO did not form colonies (Fig. 9). The total CE was significantly increased by E₂ and BP, and significantly less by P (Figs. 10 and 11a–f).

3.3. Ductulogenic capacity

Ductulogenesis was qualitatively evaluated by estimating the ability of the cells plated in collagen to form tubular and ductular structures. It was maximal in MCF-10F

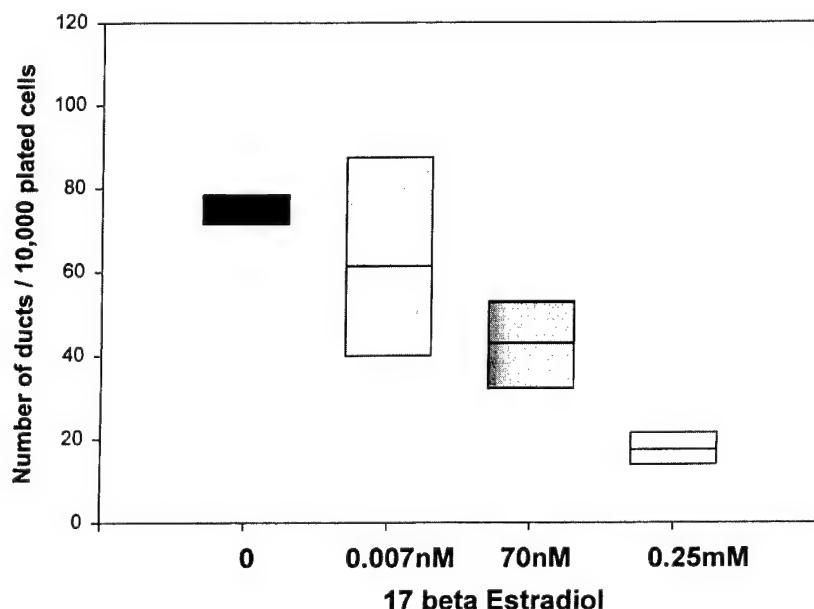


Fig. 5. Box plot showing the dose effect of 17 β -estradiol on MCF-10F cells forming ductules in collagen matrix over 10,000 cells plated.

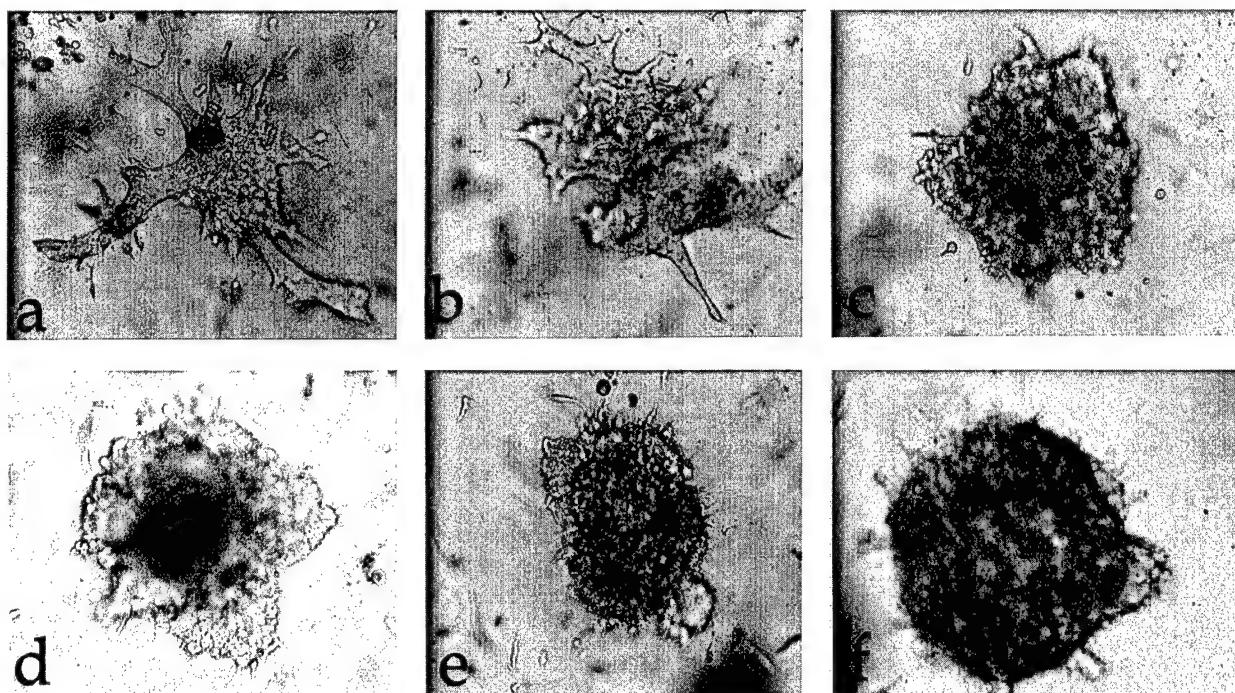


Fig. 6. (a) MCF-10F cells treated with solvent (DMSO) forming well defined ductular structures in collagen matrix; (b) 0.007 nM of E₂ induces alteration in the ductular pattern; (c) and (d) 70 nM of E₂ induces the loss of ductular formation in collagen matrix; (e) and (f) 1 µg of E₂ or BP, respectively, induces the formation of spherical masses in collagen matrix. Phase contrast microscope $\times 10$.

cells (Fig. 6a), and completely negative (–) in BP-treated cells, which grew as a solid or cystic mass. All the cells treated with E₂ exhibited decreased ability to form ductules (Fig. 6b–e). Progesterone does not affect significantly the ductulogenic capacity. The collagen matrix embedded in paraffin and cross sectioned for determination of cell

morphology showed that MCF-10F form a well-defined ductule lined by a monolayer of cuboidal epithelial cells (Fig. 12a), whereas those treated with E₂ the number of layers increase and in some cases the whole lumen is obliterated (Fig. 12b–d). BP also forms similar structures to those induced by estrogen, whereas the ductules formed by

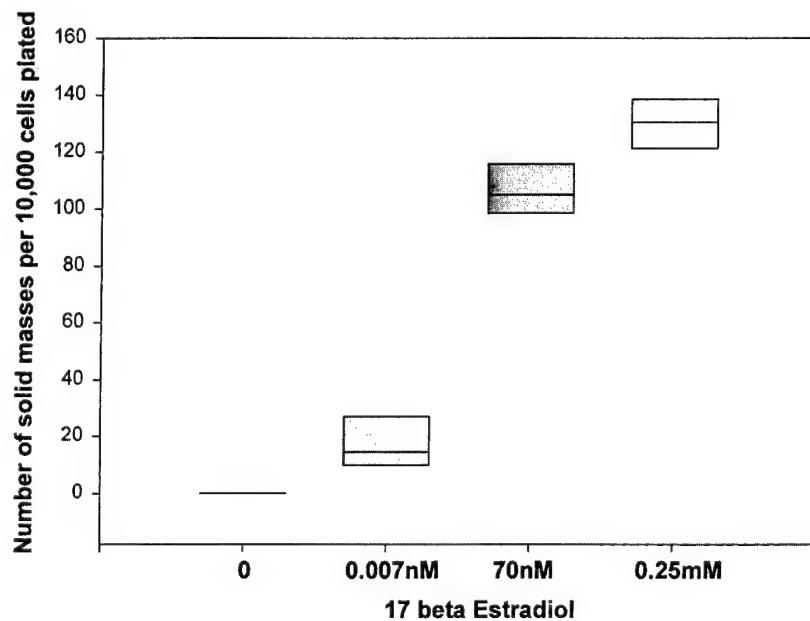


Fig. 7. Box plot showing the dose effect of 17 β -estradiol on MCF-10F cells forming solid or spherical masses in collagen matrix per 10,000 cells plated.

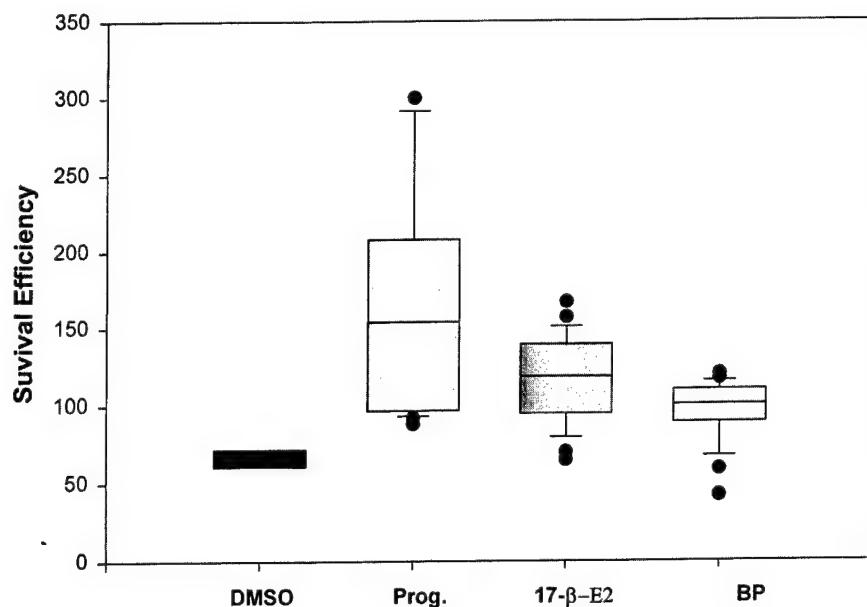


Fig. 8. Box plot showing the effect of different compounds on MCF-10F cells survival efficiency in agar methocel.

progesterone treatment are smaller with a reduced luminal size lined by a monolayer of cuboidal epithelial cells.

3.4. Genomic changes induced in E_2 and DES transformed MCF-10F cells

From the E_2 -treated cells six clones out 24 colonies were expanded and maintained in culture. These clones were designated E_2 -1– E_2 -6 (Table 2). These clones were selected for genomic analysis. DNA fingerprint analysis of parent, E_2 -, P-, and BP-treated cells and their derived clones revealed that their allelic pattern was identical in all the cell lines

analyzed. These results confirmed that all the cells tested had the same HBEC origin, and that they were free of contamination from other cell lines maintained in our laboratory.

Among 67 markers tested, which were selected based on chromosomal changes reported to be present in breast and other cancers, only clones E_2 -1 and E_2 -2, exhibited LOH in chromosome 11 (Table 1). Clones E_2 -1 and E_2 -2 identically expressed LOH in chromosome 11 at 11q23.3 (marker D11S29), and 11q24.2–25 (marker D11S912). BP-treated cells did not exhibit LOH at any of the loci tested. Interestingly, we have found that all the clones of the cells transformed with either E_2 -, BP-presented microsatellite

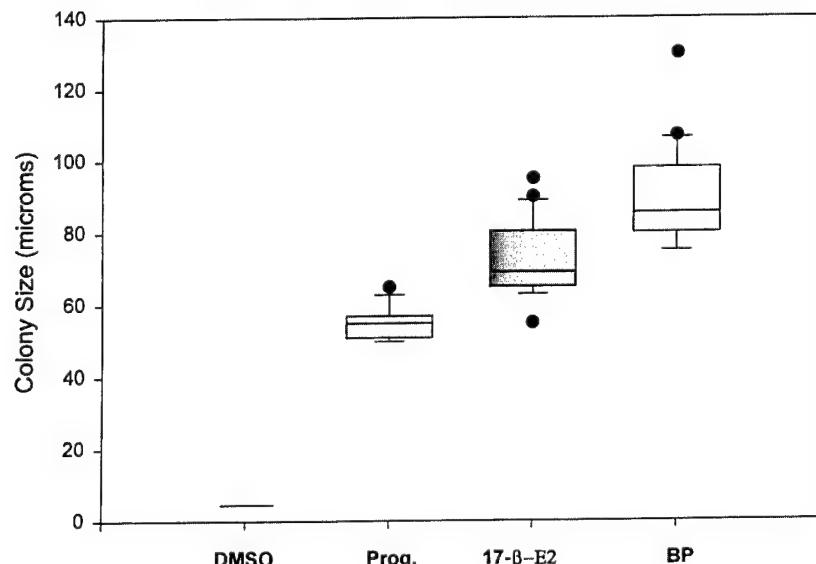


Fig. 9. Box plot showing the effect of different compounds on MCF-10F cells colony size growing in agar methocel.

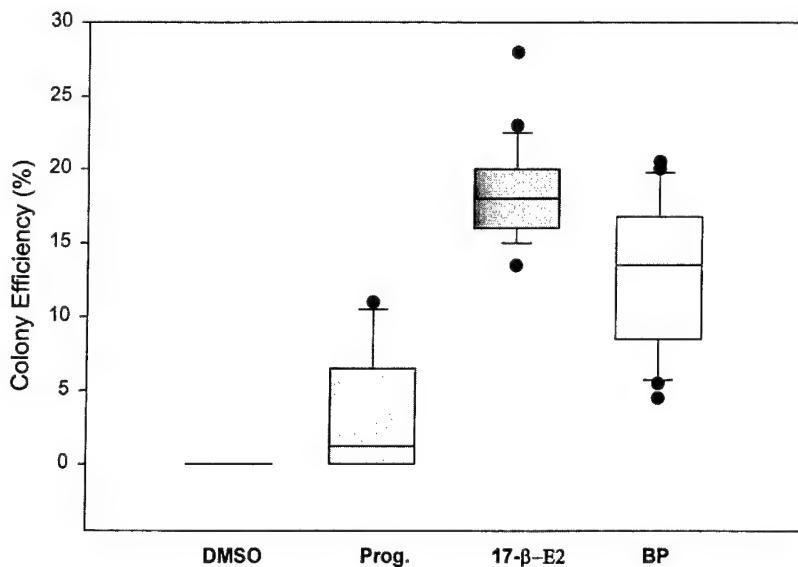


Fig. 10. Box plot showing the effect of different compounds on MCF-10F cells colony efficiency in agar methocel.

instability (MSI), expressed as an allelic expansion at 3p21 locus (marker D3S1447) (data not shown). In order to determine whether these MSIs were related to alterations in mismatch repair genes, we performed microsatellite DNA analysis in loci 1p13.1, with marker BAT40, 2p16, with marker D2S123, and 18q22.3–q23, with marker D18S58, which are related to mismatch repair genes. However, none of those markers showed alterations with this technique (Table 1).

4. Discussion

In the present work we have capitalized on the availability in our laboratory of an *in vitro* model of transformation of immortalized HBEC by the chemical carcinogen

BP for comparison with phenotypic and genomic changes induced by the natural estrogen E₂. The immortalized HBEC MCF-10F are negative for both ER- α and ER- β [42]. Short term treatment of these cells with physiological doses of E₂ induces anchorage independent growth, colony formation in agar methocel, and reduced ductulogenic capacity in collagen gel, all phenotypes whose expression is indicative of neoplastic transformation, and that are induced by BP under the same culture conditions. Progesterone was unable to induce significant increase in colony formation, although small colonies <60 mm in diameter were observed, whereas none were found in the MCF-10F cells treated with DMSO. The ductulogenic pattern was not impaired by progesterone but the luminal size was smaller than those found in the MCF-10F cells.

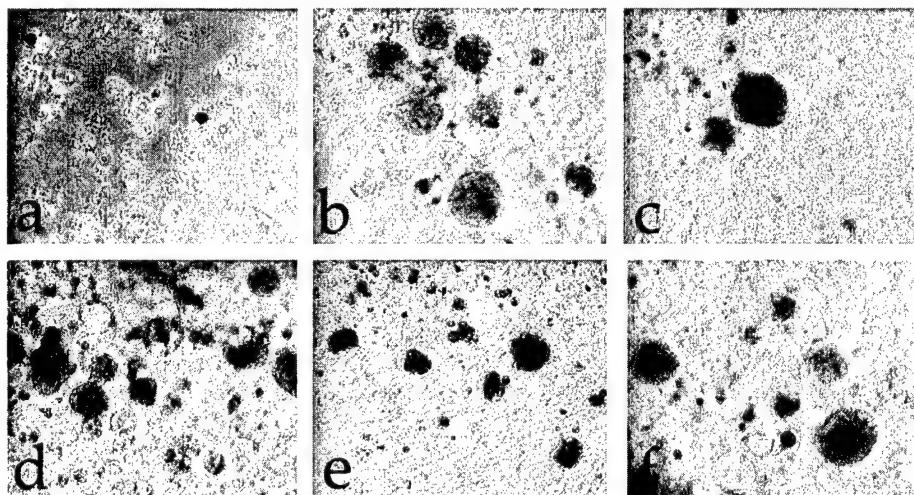


Fig. 11. MCF-10F cells plated in agar-methocel for colony assay: (a) control cells do not form colonies, only isolated cells are present; (b)–(d) colonies formed by E₂-treated MCF-10F cells at the doses of 0.007, 70 nM and 1 μ M, respectively; (e) progesterone-treated cells; (f) BP-treated cells induces slightly larger colonies. Phase contrast microscopy $\times 4$.

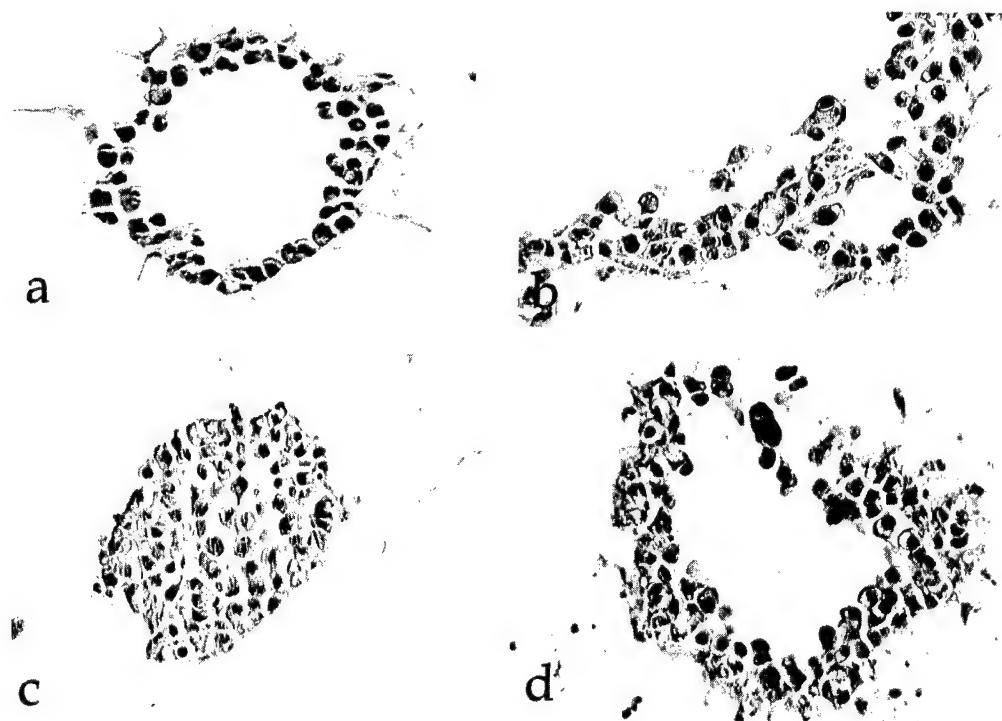


Fig. 12. Histological sections of cells growing in collagen gel. The cells have been fixed in buffered formalin, embedded in paraffin and the sections stained with hematoxylin and eosin. (a) MCF-10F cells treated with solvent (DMSO) forming well defined ductular structures lined by a single cuboidal layer of cells; (b) 0.007 nM of E₂ induces alteration in the ductular pattern forming spherical masses lined by two to three layers of cells; (c) 70 nM of E₂ induces the loss of ductular formation in collagen matrix and the solid spherical masses are composed of large cuboidal cells; (d) 1 µg of E₂ or BP induces the formation of spherical masses lined by multiple layers of cells. Phase contrast microscope $\times 10$ Hematoxylin and eosin $\times 10$.

Altogether these data clearly indicate that HBEC when treated with E₂ produces significant morphogenetic changes. The fact that the MCF-10F cells are both ER- α and ER- β negative, argue in favor of a metabolic activation of estrogens mediated by various cytochrome P450 (CYP) complexes, generating through this pathway reactive intermediates that elicit direct genotoxic effects by increasing mutation rates. An increase in CE due to either elevated rates of synthesis

or reduced rates of monomethylation will easily lead to their autoxidation to semiquinones and subsequently quinones, both of which are electrophiles capable of covalently binding to nucleophilic groups on DNA. Through this pathway estrogen and estrogen metabolites exert direct genotoxic effects that might increase mutation rates, or compromise the DNA repair system, leading to the accumulation of genomic alterations essential to tumorigenesis [18–23]. Although this

Table 2
Phenotypic markers of cell transformation induced in MCF-10F cells by E₂, and BP

Cell type	No. of passages	Doubling time (DT) (h) ^a	Colony number (CN) ^b	Colony efficiency (%) (CE) ^b	Colony size (CS) (m) ^b
MCF-10F	113	93 \pm 5.6	0.0	0.0	0.0
BP	4	42 \pm 3.8	89	18 \pm 4.5	670 \pm 46
E ₂	4	78 \pm 16.0	24 ^c	4.8 \pm 0.9	170 \pm 34
E ₂ -1 ^d	4	81 \pm 3.0	36	7.2 \pm 3.7	180 \pm 12
E ₂ -2 ^d	4	68 \pm 10	45	9.0 \pm 2.0	150 \pm 6
E ₂ -3	5	66 \pm 8.0	39	7.9 \pm 5.6	190 \pm 9
E ₂ -4	3	82 \pm 6.0	20	3.5 \pm 1.1	134 \pm 5
E ₂ -5	6	61 \pm 5.6	63	12.6 \pm 3.0	193 \pm 12
E ₂ -6	4	73 \pm 3.0	54	10.8 \pm 4.9	189 \pm 5

^a DT was determined as described in [43]. DT was significantly different by Student's *t*-test between BP and all the other cell lines ($P < 0.001$).

^b CN, CE, and CS were significantly different between MCF-10F and all other cell lines ($P = 0.00001$). CS of DES clones was significantly different from E₂ and BP cells ($P = 0.001$).

^c From 24 colonies derived from E₂-treated cells, clones E₂-1, E₂-2, E₂-3, E₂-4, E₂-5 and E₂-6 were recovered and expanded.

^d E₂-1 and E₂-2 cells have been used for detection of microsatellite DNA polymorphism.

pathway has not been demonstrated in the present work, the data are supporting but not definitively demonstrating the pathway. More studies in this subject are in progress in our laboratory to define this mechanism.

It was of great interest that by the fourth passage after four treatments during a 2-week period, clones derived from E₂-transformed cells exhibited LOH in chromosome 11, whereas during the same period of time, the chemical carcinogen BP did not induce genomic changes, even though we have previously reported that this carcinogen induces LOH in chromosome 17 [43], in addition to tumorigenesis in a heterologous host after a larger number of passages and a more prolonged selection process *in vitro* [44,45]. We have found that estrogen induces LOH in chromosome 11, as detected using the markers D11S29 and D11S912 mapped to 11q23.3 and 11q24.2–25, respectively. It has been reported that both arms of chromosome 11 contain several regions of LOH in cancers of the breast and of other organs, and that transfer of chromosome 11 to mammary cell lines suppresses tumorigenicity in athymic mice [65]. Several genes, such as *HRAs*, *CTSD*, *ILK*, *TSG101* and *KII* have been reported to be located on the short arm of chromosome 11 [53,54,65–71]. A region of deletion on 11q22–23 has been described on the long arm of chromosome 11 in 40–60% of breast tumors [51,57,59,60,72–74]. The ataxia telangiectasia susceptibility gene (*ATM*) is the most widely studied candidate gene in this region [75]. *ATM* may act upstream of the *TP53* gene in cell cycle regulation [76,77] and its heterozygous mutation is associated with high incidence of early-onset breast cancer. This region has been reported to contain several tumor suppressor genes and genes involved in the metastatic process. In this latter group, the *MMP* genes encoding matrix metalloproteases involved in invasion, *ETS1* encoding a transcription factor involved in angiogenesis, and *VACM-1*, encoding a protein probably involved in cell cycle regulation have been identified [78]. Although some of these genes might be affected during the transformation of HBEC induced by estrogens, a more detailed allelotyping using multiple markers is required for better defining the significance of LOH in these cells.

Approximately 35% of breast cancers show LOH at the D11S29 and NCAM loci [79], and a higher frequency of LOH at this locus has also been found in melanomas [80]. LOH has been found at frequencies of 25 and 29% at the distal D11S968 (11-qter) and D11S29 (11q23.3 locus), slightly above the accepted baseline of 0–20% in colorectal cancer. The fact that breast cancer, melanoma, and colorectal cancer have been found to be influenced by estrogens [81], give relevance to our data that treatment of MCF-10F cells with estrogens induces LOH in this specific locus. LOH at 11q23-qter occurs frequently in ovarian and other cancers [82,83].

The most frequent allelic loss observed in breast cancer has been reported in chromosome 17p, suggesting that genes located in that chromosome arm, such as p53 oncogene, might be a likely target for this event. [33,80–100]. We have

not been able up to now to demonstrate any LOH in chromosome 17 in estrogen transformed MCF-10F cells. However, we have used a small number of markers, and the possibility that LOH might be located at sites not tested yet cannot be ruled out. Therefore, the study of allelic imbalances at 17q and 17p, as well as in chromosome 16 [85,101,102] in estrogen transformed HBEC must be carried out to provide further understanding of the functional involvement of these chromosomes in the process of cell transformation by E₂.

The observations that E₂, and BP induce similar phenotypical, but different genomic alterations requires further investigation in order to elucidate the significance of timing of appearance of each type of changes with regards to cancer initiation and progression. There are several probable avenues for explaining these discrepancies. In this model, both estrogens and the chemical carcinogen as an early event induce phenotypic changes, whereas LOH is a rare event that is manifested in different chromosomes and only in few clones derived from E₂-treated cells. The rarity of the phenomenon is in agreement with the low frequency of LOH observed in BP transformed cells, in which the phenomenon is manifested at a more advanced stage of neoplastic progression [43,100]. Altogether these observations suggest that these two compounds might act through different genetic events for inducing similar transformation phenotypes.

Acknowledgements

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Carcinogenicity of estrogens in human breast epithelial cells¹

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Epidemiological and clinical evidences indicate that breast cancer risk is associated with prolonged ovarian function that results in elevated circulating levels of steroid hormones. Principal among these is estrogen, which is associated with two important risk factors, early onset of menarche and late menopause. However, up to now there is no direct experimental evidence that estrogens are responsible of the initiation of human breast cancer. We postulate that if estrogens are causative agents of this disease, they should elicit in human breast epithelial cells (HBEC) genomic alterations similar to those exhibited by human breast cancers, such as DNA amplification and loss of genetic material representing tumor suppressor genes. These effects could result from binding of the hormone to its nuclear receptors (ER) or from its metabolic activation to reactive metabolites. This hypothesis was tested by treating with the natural estrogen 17 β -estradiol (E₂) and the synthetic steroid diethylstilbestrol (DES) MCF-10F cells, a HBEC line that is negative for ER. Cells treated with the chemical carcinogen benzo (a) pyrene (BP) served as a positive control of cell transformation. BP-, E₂-, and DES-treated MCF-10F cells showed increases in survival efficiency and colony efficiency in agar methocel, and loss of ductulogenic capacity in collagen gel. The largest colonies were formed by BP-treated cells, becoming progressively smaller in DES- and E₂-treated cells. The loss of ductulogenic capacity was maximal in BP-, and less prominent in E₂- and DES-treated cells. Genomic analysis revealed that E₂- and DES-treated cells exhibited loss of heterozygosity in chromosomes 3 and 11, at 3p21, 3p21–21.2, 3p21.1–14.2, and 3p14.2–14.1, and at 11q23.3 and 11q23.1–25 regions, respectively. It is noteworthy that these loci are also affected in breast lesions, such as ductal hyperplasia, carcinoma in situ, and invasive carcinoma. Our data are the first ones to demonstrate that estrogens induce in HBEC phenotypic changes indicative of cell transformation and that those changes are associated with significant genomic alterations that might unravel new pathways in the initiation of breast cancer.

Key words: breast cancer; epithelial cells; estrogens; carcinogenesis.

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Epidemiological and clinical evidence indicate that breast cancer risk is associated with prolonged exposure to female ovarian hormones, mainly since a greater incidence of this disease is

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associated with early onset of menarche and late menopause, two conditions directly regulated by ovarian function (1–4). Although breast cancer is a hormone- and sex-dependent malignancy whose development is influenced by a myriad of hormones and growth factors (5, 6), estrogens have been demonstrated to be of essential importance in this phenomenon. This postulate has been further supported by the greater cancer risk observed in postmenopausal hyperestrogenism resulting from the use of estrogenic hormone replacement therapy and obesity (7, 8).

Estrogens, that are necessary for the normal development of both reproductive and non-reproductive organs, exert their physiological effects by binding to their specific receptors, the estrogen receptors (ER) α or β (9–15). ER α resides in the nucleus of target cells in an inactive form associated with a large inhibitory protein complex. Both endogenous and exogenous estrogens, such as 17 β -estradiol (E₂) and the synthetic nonsteroidal estrogen diethylstilbestrol (DES), bind to the C-terminal ligand-binding domain (LBD) of the ER α activating the receptor, which undergoes a conformational change. The activated receptor undergoes dimerization, participating in the regulation of target gene transcription by one of two mechanisms, a) binding to transcription factors, such as AP-1, forming a complex that recruits transcriptional co-activators, i.e., the steroid receptor coactivator protein 1 (SRC-1) (16), or b) the ER can form a ternary complex with a co-activator protein after its direct interaction with specific regulatory sequences within target gene promoters (9–11). Estrogens might act as well through alternate non-receptor mediated pathways. It has been recently found that overexpression of p21 in a p21-negative, ER negative cell line induced both the ER and ERE promoters in an estrogen-responsive manner. Stable p21 clones that also lack the expression of wild type ERE were responsive to the growth inhibitory effect of ICI 182,780, a potent antiestrogen, and the growth stimulatory effects of 17 β estradiol (17).

E₂, under the effect of 17 β -oxidoreductase is continuously interconverted to estrone (E₁), and both are hydroxylated at C-2, C-4, or C-16 α positions by cytochrome P450 isoenzymes, i.e., CYP1A1, CYP1A2, or CYP1B1, to form catechol estrogens (18–23). The demonstration that the catecholesterogen 4-hydroxyestradiol (4-OH-E₂) induces an estrogenic response in the uterus of ER α null mice, and the fact that this response is not inhibited by the antiestrogen ICI-182,780 (9), indicate that this catecholesterogen does not exert its effect on the ER. There is evidence as well that estrogen may not need to activate its nuclear receptors to initiate or promote breast carcinogenesis. The metabolic activation of estrogens can be mediated by various cytochrome P450 (CYP) complexes, generating through this pathway reactive intermediates that elicit direct genotoxic effects by increasing mutation rates.

An increase in CE due to either elevated rates of synthesis or reduced rates of monomethylation will easily lead to their autoxidation to semiquinones and subsequently quinones, both of which are electrophiles capable of covalently binding to nucleophilic groups on DNA via a Michael addition and thus, serve as the ultimate carcinogenic reactive intermediates in the peroxidative activation of CE. Thus, estrogen and estrogen metabolites exert direct genotoxic effects that might increase mutation rates, or compromise the DNA repair system, leading to the accumulation of genomic alterations essential to tumorigenesis (18–23). Although this pathway has been demonstrated in other systems, it still needs to be demonstrated in normal breast epithelial cells.

Breast cancers exhibit genomic alterations, such as DNA amplification and loss of genetic material that may represent tumor suppressor genes (24–39). Although their role in the causation of the disease has not been clearly established, it is generally accepted that the accumulation of genetic alterations promotes tumor progression (38, 39). Specific types of genetic alterations, then, might identify essential steps in the initiation and/or progression of cancer. We postulate that if estrogens initiate the neoplastic process or are responsible for its progression, they would induce in the normal breast epithelium the same type of genomic alterations observed in spontaneous malignancies. In order to test this hypothesis we evaluated the transforming potential of E₂ and DES on human breast epithelial cells (HBEC) in vitro, utilizing the spontaneously immortalized HBEC MCF-10F (40, 41). This cell line lacks both ER- α and ER- β , although this latter receptor is induced in cells transformed by chemical carcinogens (42). The same phenotypes and characteristics that were expressed by MCF-10F cells transformed by the chemical carcinogen benz(a)pyrene (BP) and oncogenes (43–46) were evaluated in E₂ and DES treated cells: anchorage independent growth, colony formation in agar methocel, ductulogenic capacity in collagen gel, and invasiveness index in Matrigel. In addition, DNA of treated cells was analyzed for specific genomic alterations such as loss of heterozygosity (LOH) at chromosomal loci known to be affected in spontaneously occurring breast lesions, such as

ductal hyperplasia, carcinoma *in situ*, and invasive carcinoma (47-60).

MATERIAL AND METHODS

Cells and dose selection

MCF-10F cells at passage 113 were cultured in DMEM:F-12 medium containing 1.05 mM calcium (Ca^{2+}), antibiotics, antimycotics, hormones, growth factors, and equine serum as previously described (44). In order to determine the optimal doses for the expression of the cell transformation phenotype we treated the immortalized human breast epithelial cells (HBEC) MCF-10 F with 17 β -estradiol (E_2) for testing whether they express colony formation in agar methocel, or colony efficiency (CE), and loss of ductulogenesis in collagen matrix, phenotypes also induced by the carcinogen benz (a) pyrene (BP) (44, 45). MCF-10F cells were treated with 0.0, 0.07 nM, 70 nM, or 0.25 mM of E_2 twice a week for two weeks. CE increased from 0 in controls to 6.1, 9.2, and 8.7 with increasing E_2 doses. Ductulogenesis was 75 ± 4.9 in control cells; it decreased to 63.7 ± 28.8 , 41.3 ± 12.4 , and 17.8 ± 5.0 in E_2 treated cells, which also formed solid masses, whose numbers increased from 0 in controls to 18.5 ± 6.7 , 107 ± 11.8 and 130 ± 10.0 for each E_2 dose. Based upon these results a dose of 3.7 μM (1 $\mu\text{g}/\text{ml}$) was selected for testing the effect of E_2 or DES.

Evaluation of the effect of estrogens on the expression of cell transformation phenotypes

The spontaneously immortalized MCF-10F cells, treated cells and derived clones were maintained in DMEM:F-12 (1:1) medium with a 1.05 mM Ca^{2+} concentration. All cell lines were regularly tested for correct identity using a fingerprint cocktail of three minisatellite plasmid probes (ATCC, Rockville, MD). Culture media were prepared by the Central Center Tissue Culture Facility at the Fox Chase Cancer (Philadelphia, PA). MCF-10F cells were treated with 1.0 $\mu\text{g}/\text{ml}$ E_2 (Aldrich, St. Louis, MO) or DES (Sigma Chemical Co., St. Louis, MO); control cells were treated with DMSO. MCF-10F cells treated with 1.0 $\mu\text{g}/\text{ml}$ benz (a) pyrene (BP) served as positive controls for cell transformation assays. In order to mimic the intermittent exposure of HBEC to endogenous estrogens, all cells were first treated with E_2 , DES, or BP at 72 hrs and 120 hours post plating. At the end of the first week of treatment, the cells were divided for evaluation of specific phenotypic characteristics or they were passaged for administration of another two periods of hormonal treatment. Treatments were repeated during the second week, and cells were collected at the 14th day for phenotypic and genotypic analysis (Fig. 1). At the end of each treatment period the culture medium was replaced with fresh medium.

At the end of the second week of treatment the cells were assayed for determination of doubling time (DT), survival efficiency (SE), colony efficiency (CE), colony size (CS), and ductulogenic capacity, as described in previous publications (44, 45).

Colony formation in agar-methocel assay

This technique was utilized as an *in vitro* assay for anchorage independent growth, a parameter indicative of transformation. Parental, control, and treated cells were suspended at a density of 2×10^4 cells/ml in 2 ml of 0.8% methocel (Sigma Co, St. Louis, MO) dissolved in DMEM:F-12 (1:1) medium containing 20% horse serum. Cells from each treatment group and time point were plated in four 24-well chambers pre-coated with 0.5 ml of 0.8% agar base in DMEM: F-12 medium, which was replaced with fresh feeding medium containing 0.8% methocel twice a week. The actual number of cells plated was calculated as the average of cells counted at 10 \times magnification in 5 individual fields, and multiplied by a factor of 83. CE and CS were measured 21 days after plating. CE was determined by a count of the number of colonies greater than 100 μm in diameter, and expressed as a percentage of the original number of cells plated per well.

Ductulogenesis in collagen matrix

This *in vitro* technique evaluates the capacity of cells to differentiate by providing evidence of whether treated cells form tridimensional structures when grown in a collagen matrix. Parental, control, and treated cells were suspended at a final density of 2×10^3 cells/ml in 89.3% Vitrogen¹⁰⁰ collagen matrix (Collagen Co., Palo Alto, CA) and plated into four 24 well chambers pre-coated with agar base. The cells were fed fresh feeding medium containing 20% horse serum twice a week. The cells were examined under an inverted microscope for a period of 21 days or longer for determining whether they formed ductule-like structures or whether they grew as unorganized clumps. The final structures were photographed, and then fixed in 10% neutral buffered formalin, embedded in paraffin, sectioned, and stained with hematoxylin-eosin for histological examination. Immunohistochemical techniques were utilized for detecting the expression of specific differentiation genes.

Genomic analysis of treated cells

DNA isolation. To obtain DNA, treated and control cells were lysed in 5ml of TNE (0.5M Tris pH 8.9, 10mM NaCl, 15-mM EDTA) with 500 $\mu\text{g}/\text{ml}$ proteinase K and 1% sodium dodecyl sulfate (SDS), and incubated at 48°C for 24 h. Following two extractions with phenol (equilibrated with 0.1 M Tris pH 8.0), the DNA was spooled from 2 volumes of 100% ethanol, air dried and resuspended in 20 mM EDTA. The DNA was then treated sequentially with RNase A (100 $\mu\text{g}/\text{ml}$) for 1 hour at 37°C and 100 $\mu\text{g}/$

TABLE 1. *Microsatellite DNA Polymorphism Analysis of MCF-10F Cells Treated with 17 β -Estradiol (E2), Diethylstilbestrol (DES), or Benz(a)pyrene (BP)*

Ch	Marker	Location	MCF10-F	E2-1	E2-2	DES-1	DES-3	DES-4	DES-5	BP
1	D1S104	1p21-1p23	○	○	○	○	○	○	○	○
1	BAT-40	1p13.1	○	○	○	○	○	○	○	○
2	D2S171	2p24-21	○	○	○	○	○	○	○	○
2	D2S123	2p16	○	○	○	○	○	○	○	○
3	D3S1297	○	○	○	○	○	○	○	○	○
3	D3S1560	3p26-3p25	○	○	○	○	○	○	○	○
3	D3S1304	3p26-3p25	○	○	○	○	○	○	○	○
3	D3S1307	3p26-p25	○	○	○	○	○	○	○	○
3	D3S1289	3p23-3p21	○	○	○	○	○	○	○	○
3	D3S1449	3p22.3-3p21.3	○	○	○	○	○	○	●	○
3	D3S1478	3p21.3-21.2	○	○	○	○	○	○	●	○
3	D3S2384	3p21.3-21.2	○	○	○	○	○	○	●	○
3	D3S1450	3p21.1-3p14.2	○	○	○	○	○	○	●	○
3	D3S1217	3p21	○	○	○	○	○	○	●	○
3	D3S1447	3p21	○	○	○	○	○	○	○	○
3	D3S1241	3p21	○	○	○	○	○	○	○	○
3	D3S1448	3p21	○	○	○	○	○	○	○	○
3	D3S1480	3p14	○	○	○	○	○	○	○	○
6	ESR	6q24-27	○	○	○	○	○	○	○	○
8	MYCL-1	8q24.1	○	○	○	○	○	○	○	○
9	D9S199	9p23	○	○	○	○	○	○	○	○
9	D9S157	9p23-22	○	○	○	○	○	○	○	○
9	D9S171	9p21	○	○	○	○	○	○	○	○
9	D9S165	9p21	○	○	○	○	○	○	○	○
11	D11S988	1pter-qter	○	○	○	○	○	○	○	○
11	D11S922	11p15.5	○	○	○	○	○	○	○	○
11	H-RAS1	11p15.5	○	○	○	○	○	○	○	○
11	CCKBR	11p15.4	○	○	○	○	○	○	○	○
11	D11S1392	11p13	○	○	○	○	○	○	○	○
11	Int-2	11q13	○	○	○	○	○	○	○	○
11	D11S907	11p13	○	○	○	○	○	○	○	○
11	D11S911	11q13-11p23	○	○	○	○	○	○	○	○
11	D11S436	11p12-11p11.1	○	○	○	○	○	○	○	○
11	D11S614	11q22-11q23	○	○	○	○	○	○	○	○
11	D11S940	11q22	○	○	○	○	○	○	○	○
11	DRD2	11q23.1	○	○	○	○	○	○	○	○
11	D11S968	11q23.1-11q25	○	○	○	○	○	○	○	○
11	D11S29	11q23.3	●	●	○	○	○	○	○	○
11	D11S925	11q23.3-11q24	○	○	○	○	○	○	○	○
11	D11S912	11q24.2-11q25	●	●	○	○	○	○	○	○
12	IGF-1	12q22-12q23	○	○	○	○	○	○	○	○
13	D13S289	13q12.2	○	○	○	○	○	○	○	○
13	D13S260	13q12.3	○	○	○	○	○	○	○	○
13	D13S267	13q12.3	○	○	○	○	○	○	○	○
13	D13S171	13q12.3-13	○	○	○	○	○	○	○	○
13	D13S218	13q13-14.1	○	○	○	○	○	○	○	○
13	GABRB-1	13q14.2	○	○	○	○	○	○	○	○
13	D13S155	13q14.3-21.2	○	○	○	○	○	○	○	○
16	D16S540	○	○	○	○	○	○	○	○	○
17	D17S849	17p13.3	○	○	○	○	○	○	○	○
17	D17S796	17p13.1	○	○	○	○	○	○	○	○
17	D17S513	17p13.1	○	○	○	○	○	○	○	○
17	Tp53	17p13.1	○	○	○	○	○	○	○	○
17	D17S786	17p13.1	○	○	○	○	○	○	○	○
17	D17S793	17p13.1-17p11.2	○	○	○	○	○	○	○	○

TABLE 1 (contd)

Ch	Marker	Location	MCF10-F	E2-1	E2-2	DES-1	DES-3	DES-4	DES-5	BP
17	D17S945	17p13-12	○	○	○	○	○	○	○	○
17	D17S520	17p12	○	○	○	○	○	○	○	○
17	D17S800	17q11.1-12	○	○	○	○	○	○	○	○
17	THRA-1	17q11.2-12	○	○	○	○	○	○	○	○
17	D17S787	17q21-22	○	○	○	○	○	○	○	○
17	D17S855	17q21.2	○	○	○	○	○	○	○	○
17	D17S1323	17q21.2	○	○	○	○	○	○	○	○
17	D17S808	17q23.2	○	○	○	○	○	○	○	○
17	D17S789	17q24	○	○	○	○	○	○	○	○
17	D17S515	17q24.2-25.2	○	○	○	○	○	○	○	○
17	D17S785	17q25.2	○	○	○	○	○	○	○	○
18	D18S58	18q22.3-23	○	○	○	○	○	○	○	○

E₂, 17 β -estradiol; DES, diethylestilbestrol; BP, benzo(a)pyrene; Ch, chromosome.

ml proteinase K, 1% SDS, at 48°C for 3 h, followed by two extractions with saturated phenol. The DNA was again retrieved from the aqueous phase by ethanol precipitation, washed extensively in 70% ethanol, and after air-drying suspended in TE (10 mM Tris, pH8.0), 1 mM EDTA.

Detection of allelic loss. We evaluated for allelic losses the regions of chromosomes 1, 2, 3, 6, 8, 9, 11, 12, 13, 16, 17, and 18 most frequently reported to exhibit loss of heterozygosity (LOH) in spontaneous breast tumors (Table 1). DNA amplification of microsatellite length polymorphisms was utilized for detecting allelic losses present in the transformed clones. Microsatellites are polymorphic markers used primarily for gene mapping which can be broadly defined as relatively short (<100 bp) runs of tandem repeated di- to tetranucleotide sequence motifs (61-63). The origin and nature of these polymorphism sequences is not well established, but they may result from errors of the polymerase during replication and/or from slightly unequal recombination between homologous chromatids during meiosis. These microsatellites have proven to be useful markers for investigating LOH and could be applicable to allelotyping as well as regional mapping of deletions in

specific chromosomal regions. They are highly polymorphic, very common (between 10⁵ and 10⁶ per genome), and are flanked by unique sequences that can serve as primers for polymerase chain reaction (PCR) amplification (64).

DNA fingerprinting. Before performing DNA amplification of microsatellite DNA polymorphisms to detect allelic losses present in E₂-, DES-, and BP-treated cells, we verified by DNA fingerprinting whether all the clones derived from MCF-10F treated cells were from the same lineage. Genomic DNA was extracted from the cells listed in Table 1. The identity of these cells was confirmed by Southern blot hybridization of genomic DNA with a cocktail of the three minisatellite probes D2S44, D14S13 and D17S74. Genomic DNAs were digested with HinfI, and hybridized with probes under standard condition (64).

PCR analysis of microsatellites. Primers used for the analysis of microsatellite polymorphisms are given elsewhere (64). Conditions for PCR amplification were as follows: 30 ng of genomic DNA, 100 pmoles of each oligonucleotide primer, 1×PCR buffer (Perkin Elmer Cetus), 5 μ M each of TTP, dCTP, dGTP, and dATP, 1 μ Ci (³²P) dATP (300 mCi/mmol)

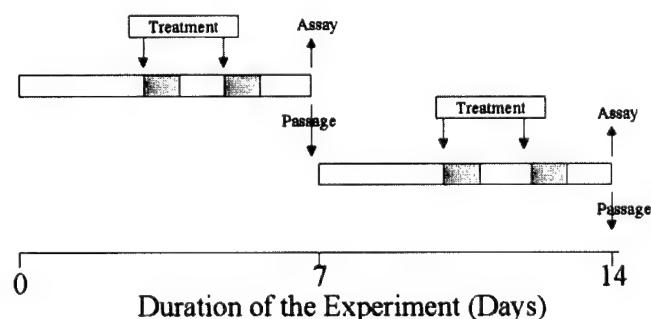


Fig. 1. MCF-10F cells were treated with E₂, DES, or BP at 72 hrs and 120 hours post plating. Treatments were repeated during the second week, and cells were collected at the 14th day for phenotypic and genotypic analysis.

(Dupont, NEN, Boston, MA), and 0.5 units of AmpliTaq DNA polymerase (Perkin Elmer Cetus) in 10 ml volumes. The reactions were processed through 27 cycles of 1 min at 94°C, 1 min at the appropriate annealing temperatures determined for each set of primers, and 1 min at 72°C; with a final extension of 7 min at 72°C. Reaction products were diluted 1:2 in loading buffer (90% formamide, 10 mM EDTA, 0.3% bromophenol blue, and 0.3% xylene cyanol), heated at 90°C for 5 min and loaded (4ml) onto 5% to 6% denaturing polyacrylamide gels. After electrophoresis, gels were dried at 70°C and exposed to XAR-5 film with a Lightning Plus intensifying screen at -80°C for 12 to 24 h. Allele sizes were determined by comparison to M13mp18 sequencing ladders.

Detection of allelic loss. LOH was defined as a total loss of, or a 50%, or more reduction in density in one of the heterozygous alleles. All experiments were repeated at least three times to avoid false positive or false negative results. To control for possible DNA degradation, the same blots used to assess allelic loss were analyzed with additional DNA gene probes that detect large fragments. The bands were quantitated using a UltraScan XL laser densitometry (Pharmacia LKB Biotechnology Inc.) within the linear range of the film.

RESULTS

Effect of estrogens on the expression of transformation phenotypes

The doubling time (DT) of MCF-10F cells was 93 ± 5.6 hours. It was decreased, but no significantly, in E₂- and DES-treated cells and their derived clones. A significant decrease, greater than 50%, was observed in BP-treated cells (Table 2). Evaluation of colony formation at the end of the second week of treatment revealed that MCF-10F cells treated with E₂, DES, or BP, formed colonies in agar-methocel, whereas MCF-10F control cells treated with DMSO did not (Table 2, Fig. 2). E₂ treated cells formed 24 colonies, from which six clones were expanded and maintained in culture. These clones were designated E₂-1 to E₂-6 (Table 2). DES treated MCF-10F cells formed 151 colonies, from which 24 colonies were isolated and seven clones survived. They were expanded and maintained in culture, being designated DES-1 to DES-7 (Table 2). BP treated cells formed 89 colonies, which had an average size of 670 ± 46 μ m in diameter. Those colonies formed by DES- and E₂-treated cells and their respective

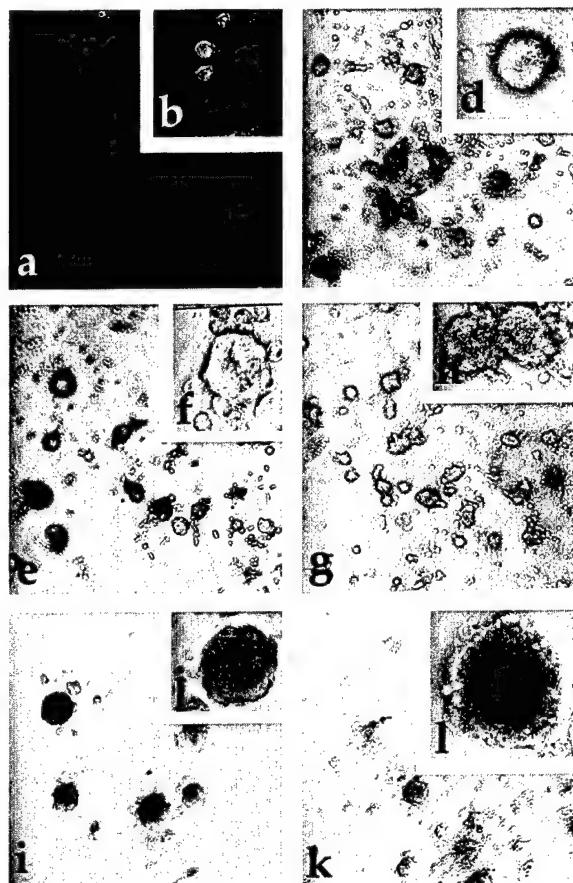


Fig. 2. MCF-10F cells plated in agar-methocel for colony assay. Control cells do not form colonies. Only isolated cells are present (a, 4 \times), (b, 10 \times). (c-k), colonies formed by E₂-, DES, and BP-treated MCF-10F cells. E₂-induced colonies (c, 4 \times) (d, 10 \times). DES-induced colonies (e, 4 \times); (f, 10 \times). Colonies of E₂-2 clone (g, 4 \times) (h, 10 \times). Colonies of DES-3 clone (i, 4 \times) (j, 10 \times). Colonies of BP-treated cells (k, 4 \times) (l, 10 \times). Phase contrast.

derived clones were significantly smaller than those formed by BP transformed cells. However, DES-treated cell colonies were larger than those formed by E₂-treated cells and their derived clones (Table 2, Fig. 2).

Ductulogenesis was qualitatively evaluated by estimating the ability of the cells plated in collagen to form tubular and ductular structures. It was maximal (++) in MCF-10F cells, and completely negative (-) in BP-treated cells, which grew as a solid or cystic mass (Table 2). E₂-, DES-treated cells, and E₂-4 and DES-1 clones exhibited a moderately decreased ability to form ductules (+). It was interesting to observe that all the other clones derived from E₂- and DES-

TABLE 2. *Phenotypic markers of cell transformation induced in MCF-10F cells by 17 β estradiol (E₂), Diethylstilbestrol (DES) and Benz (a) pyrene (BP)*

Cell Type	No. of Passages	Doubling time (DT) ^a	Colony Number (CN) ^b	Colony Efficiency (%) CE) ^c	Colony Size (CS) (μ m) ^d	Ductulogenesis ^g
MCF-10F	113	93 \pm 5.6	0.0	0.0	0.0	++
BP	4	42 \pm 3.8	89	18 \pm 4.5	670 \pm 46	-
E ₂	4	78 \pm 16.0	24 ^e	4.8 \pm 0.9	170 \pm 34	+
DES	4	73 \pm 13	151 ^f	30.20 \pm 8.9	190 \pm 23	+
E ₂ -1*	4	81 \pm 3.0	36	7.2 \pm 3.7	180 \pm 12	+/-
E ₂ -2*	4	68 \pm 10	45	9.0 \pm 2.0	150 \pm 6	+/-
E ₂ -3	5	66 \pm 8.0	39	7.9 \pm 5.6	190 \pm 9	-
E ₂ -4	3	82 \pm 6.0	20	3.5 \pm 1.1	134 \pm 5	+
E ₂ -5	6	61 \pm 5.6	63	12.6 \pm 3.0	193 \pm 12	+/-
E ₂ -6	4	73 \pm 3.0	54	10.8 \pm 4.9	189 \pm 5	-
DES-1*	4	73 \pm 9	167	33.17 \pm 6.3	278 \pm 40	+
DES-2	4	74 \pm 10	148	29.4 \pm 10.0	189 \pm 23	+/-
DES-3*	3	78 \pm 5	130	25.8 \pm 3.9	278 \pm 12	+/-
DES-4*	3	75 \pm 8	189	37.5 \pm 7.3	239 \pm 34	+/-
DES-5*	6	68 \pm 10	167	33.17 \pm 5.9	360 \pm 60	-
DES-6	5	67 \pm 3	150	29.8 \pm 10	290 \pm 32	+/-
DES-7	5	78 \pm 4	99	19.6 \pm 6.9	207 \pm 28	+/-

^a Doubling time (DT) in hours, was determined as described in (43). DT was significantly different by Student's t-test between BP and all the other cell lines ($p<0.001$). ^b Colony number (CN); ^c Colony efficiency (CE), and ^d Colony size (CS). These three parameters were significantly different between MCF-10F and all other cell lines ($p=0.00001$). CS of DES clones was significantly different from E₂ and BP cells ($p=0.001$). ^e From 24 colonies derived from E₂-treated cells, clones E₂-1, E₂-2, E₂-3, E₂-4, E₂-5 and E₂-6 were recovered and expanded. ^f From 151 colonies derived from DES treated cells, 24 colonies were isolated and clones DES-1, 2, 3, 4, 5, 6 and 7 were recovered and expanded. ^gDuctulogenesis, duct-like formation in collagen gel. * These cells have been used for detection of microsatellite DNA polymorphism.

treated cells exhibited an overall decrease of ductulogenic capacity. Clones E₂-3, E₂-6, and DES-5 had completely lost this property, being in this sense similar to BP-treated cells (Table 2).

Genomic changes induced in E₂ and DES transformed MCF-10 cells

DNA fingerprint analysis of parent, E₂-, DES-, and BP-treated cells and their derived clones revealed that their allelic pattern was identical in all the cell lines analyzed (Fig. 3). These results confirmed that all the cells tested had the same HBEC origin, and that they were free of contamination from other cell lines maintained in our laboratory.

Among 67 markers tested, which were selected based on chromosomal changes reported to be present in breast and other cancers, only clones DES-5, E₂-1 and E₂-2, exhibited LOH in chromosomes 3 and 11, respectively (Table 1). LOH in chromosome 3 was detected at three different loci, which were detected with five different markers, 3p21.3-21.2 (marker D3S1478

and D3S2384), 3p21.1-14.2 (marker D3S1450), and 3p21 (marker D3S1217 and D3S1447) (Fig. 4, Table 1). It was of interest that clone DES-5, in addition to exhibiting LOH in chromosome 3, was the one exhibiting the most marked expression of transformation phenotypes, i.e., larger colony size and absent ductulogenic ability in collagen gel (Table 2). Clones E₂-1 and E₂-2 identically expressed LOH in chromosome 11 at 11q23.3 (marker D11S29), and 11q24.2-q25 (marker D11S912). BP-treated cells did not exhibit LOH at any of the loci tested. Interestingly, we have found that all the clones of the cells transformed with either E₂, DES or BP presented microsatellite instability (MSI), expressed as an allelic expansion at 3p21 locus (marker D3S1447) (data not shown). In order to determine whether these MSIs were related to alterations in mismatch repair genes, we performed microsatellite DNA analysis in loci 1p13.1, with marker BAT40, 2p16, with marker D2S123, and 18q22.3-23, with marker D18S58, which are related to mismatch repair genes.

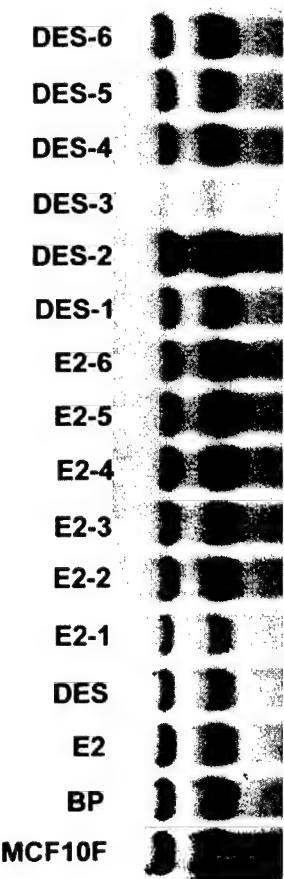


Fig. 3. Fingerprint analysis of genomic DNA obtained from the cells listed in table 2 using markers listed in Materials and Methods.

However, none of those markers showed alterations with this technique (Table 1).

DISCUSSION

In the present work we have capitalized on the availability in our laboratory of an in vitro model of transformation of immortalized HBEC by the chemical carcinogen BP for comparison with phenotypic and genomic changes induced by the natural estrogen 17 β -estradiol (E₂) and the synthetic estrogen diethylstilbestrol (DES) in the same cells (44, 45). The immortalized human breast epithelial cells MCF-10F are negative for both ER- α and ER- β (42). Short term treatments of these cells with these two estrogenic compounds induce anchorage independent growth, colony formation in agar methocel, and reduced ductulogenic capacity in collagen gel, all pheno-

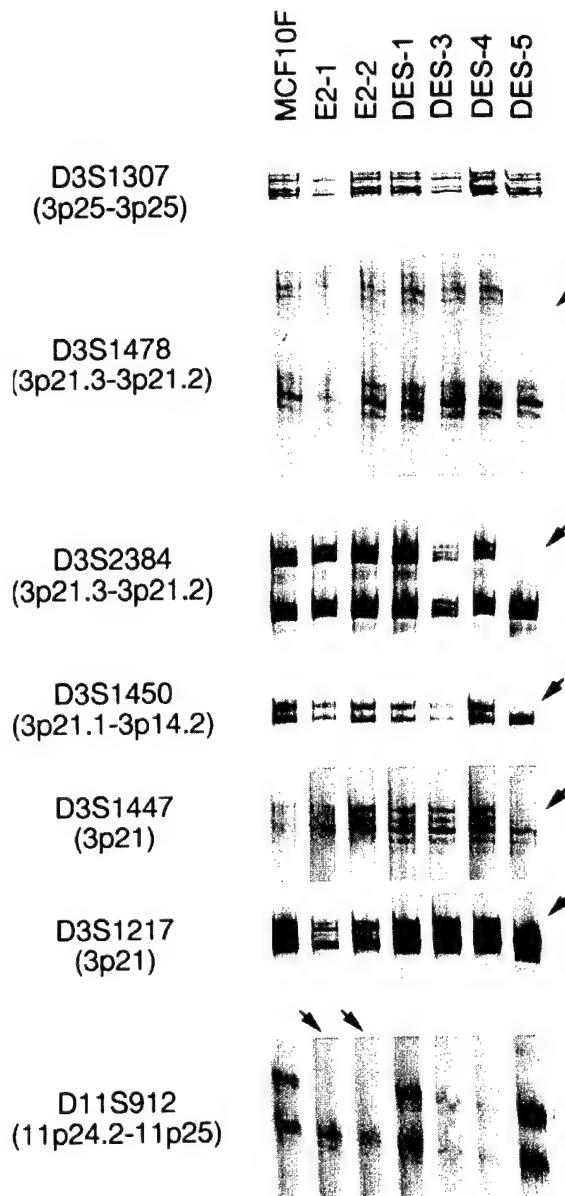


Fig. 4. LOH analysis of MCF-10F, and of clones E2-1, E2-2, and DES-1, DES-3, DES-4, and DES-5, derived from E₂ and DES-treated cells. Arrows indicate the loss of alleles in E₂-1, E₂-2, and DES-5 clones.

types whose expression is indicative of neoplastic transformation, and that are induced by BP under the same culture conditions. It was of great interest that by the fourth passage after 4 treatments during a two-week period, clones derived from DES- and E₂-transformed cells exhibited loss of heterozygosity in chromosomes 3 and 11, respectively, whereas during the same period of time the chemical carcinogen BP did not induce genomic changes, even though we have previously re-

ported that this carcinogen induces LOH in chromosome 17 (43), in addition to tumorigenesis in a heterologous host after a larger number of passages and a more prolonged selection process *in vitro* (44, 45). The expression of LOH in chromosome 3 in DES-transformed breast epithelial cells acquired relevance in view of the light that frequent homozygous deletions, rearrangements, and hypermethylation at 3p21 loci have been reported to be present in spontaneously occurring breast lesions, such as ductal hyperplasia, carcinoma *in situ*, and invasive carcinoma (31, 47–50, 65–68). The existence of suppressor genes on 3p has also been suggested by transfection studies in which 3p DNA fragments inhibited tumorigenesis in nude mice (68, 69). We have observed LOH at 3p21 using markers D3S1217 and D3S1447, and in the region 3p21.1-p21.2 with markers D3S1478 and D3S2384. LOH in this region has been reported in nearly all-small cell lung carcinomas (70). Even though deletion in these regions is not considered to be specific for breast cancer, our observations might indicate that they represent a genetic event triggered by estrogens, which could play a key role in the development and progression of tumors originated from this type of epithelium. LOH in 3p21.3 has been found more frequently in breast cancer metastases than in primary tumors. Several putative "metastasis-related genes" are located in this region, such as 37LRP (71–74), CTNNB1, that encodes β catenin (75), and the α RLC gene, that encodes a new integrin subunit, identified by positional cloning, that shows homology with the $\alpha 1$ integrin involved in the metastatic process (70). We have also found LOH in the 3p21.1–14.2 (marker D3S1450). This region has been found to be associated with dysregulated cell proliferation rather than with tumor progression (50). It is also frequently deleted in *in situ* carcinoma, benign tumors, and familial breast cancers (51, 76, 77). The 3p14.2 region contains a fragile site known as FRA3B, from which the FHIT gene has recently been cloned. It encodes a protein showing homology with a yeast hydrolase, and its transcripts show rearrangements in different cell lines and tumors (52, 77). Recently, telomerase-regulating genes have been located in 3p21.3-p22 and 3p12–21.1 using the microcell monochromosome transfer technique (78). We have also found that estrogen induces LOH in chromosome 11, as detected using the

markers D11S29 and D11S912 mapped to 11q23.3 and 11q24.2–25, respectively. It has been reported that both arms of chromosome 11 contain several regions of LOH in cancers of the breast and of other organs, and that transfer of chromosome 11 to mammary cell lines suppresses tumorigenicity in athymic mice (79). Several genes, such as HRAs, CTSD, ILK, TSG101 and KI1 have been reported to be located on the short arm of chromosome 11 (53–54, 79–85). A region of deletion on 11q22–23 has been described on the long arm of chromosome 11 in 40 to 60% of breast tumors (51, 57, 59, 60, 86, 88). The ataxia telangiectasia susceptibility gene (ATM) is the most widely studied candidate gene in this region (89). ATM may act upstream of the TP53 gene in cell cycle regulation (90, 91) and its heterozygous mutation is associated with high incidence of early-onset breast cancer. This region has been reported to contain several tumor suppressor genes and genes involved in the metastatic process. In this latter group, the MMP genes encoding matrix metalloproteases involved in invasion, ETS1 encoding a transcription factor involved in angiogenesis, and VACM-1, encoding a protein probably involved in cell cycle regulation have been identified (92). Although some of these genes might be affected during the transformation of HBEC induced by estrogens, a more detailed allelotyping using multiple markers is required for better defining the significance of LOH in these cells.

Approximately 35% of breast cancers show LOH at the D11S29 and NCAM loci (93), and a higher frequency of LOH at this locus has also been found in melanomas (94). LOH has been found at frequencies of 25% and 29% at the distal D11S968 (11qter) and D11S29 (11q23.3 locus), slightly above the accepted baseline of 0–20 per cent in colorectal cancer. The fact that breast cancer, melanoma, and colorectal cancer have been found to be influenced by estrogens (95), give relevance to our data that treatment of MCF-10F cells with estrogens induces LOH in this specific locus. LOH at 11q23-qter occurs frequently in ovarian and other cancers (96, 97).

The most frequent allelic loss observed in breast cancer has been reported in chromosome 17p, suggesting that genes located in that chromosome arm, such as p53 oncogene, might be a likely target for this event. (33, 93–114). We have not been able up to now to demonstrate

any LOH in chromosome 17 in estrogen transformed MCF-10F cells. However, we have used a small number of markers, and the possibility that LOH might be located at sites not tested yet cannot be ruled out. Therefore, the study of allelic imbalances at 17q and 17p, as well as in chromosome 16 (99, 115, 116) in estrogen transformed HBEC must be carried out to provide further understanding of the functional involvement of these chromosomes in the process of cell transformation by E₂ and DES.

The observations that E₂, DES, and BP induce similar phenotypical, but different genomic alterations requires further investigation in order to elucidate the significance of timing of appearance of each type of changes with regards to cancer initiation and progression. There are several probable avenues for explaining these discrepancies. In this model, phenotypical changes are induced by both estrogens and the chemical carcinogen as an early event, whereas LOH is a rare event that is manifested in different chromosomes and only in few clones derived from E₂ and DES treated cells. The rarity of the phenomenon is in agreement with the low frequency of LOH observed in BP transformed cells, in which the phenomenon is manifested at a more advanced stage of neoplastic progression (43, 114). Altogether these observations suggest that these three compounds might act through different genetic events for inducing similar transformation phenotypes.

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Neoplastic Transformation of Human Breast Epithelial Cells by Estrogens and Chemical Carcinogens

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Sporadic breast cancer, the most common cancer diagnosed in American and Northern European women, is gradually increasing in incidence in most Western countries. Prevention would be the most efficient way of eradicating this disease. This goal, however, cannot be accomplished until the specific agent(s) or mechanisms that initiate the neoplastic process are identified. Experimental studies have demonstrated that mammary cancer is a hormone-dependent multistep process that can be induced by a variety of compounds and mechanisms, that is, hormones, chemicals, radiation, and viruses, in addition to or in combination with genetic factors. Although estrogens have been shown to play a central role in breast cancer development, their carcinogenicity on human breast epithelial cells (HBECs) has not yet been clearly demonstrated. Breast cancer initiates in the undifferentiated lobules type 1, which are composed of three cell types: highly proliferating cells that are estrogen-receptor negative (ER-), nonproliferating cells that are ER positive (ER+), and very few (<1%) ER+ cells that proliferate. Interestingly, endogenous 17 β -estradiol (E₂) is metabolized by the cytochrome P450 enzyme isoforms CYP1A1 and

CYP1B1, which also activate benzo[a]pyrene (B[a]P), a carcinogen contained in cigarette smoke. We postulate that if estrogens are carcinogenic in HBECs, they should induce the same transformation phenotypes induced by chemical carcinogens and ultimately genomic changes observed in spontaneously developing primary breast cancers. To test this hypothesis we compared the transforming potential of E₂ on the HBEC MCF-10F with that of B[a]P. Both E₂ and B[a]P induced anchorage-independent growth, colony formation in agar methocel, and loss of ductulogenic capacity in collagen gel, all parameters indicative of cell transformation. In addition, the DNA of E₂-transformed cells expressed LOH in chromosome 11 at 11q23.3, 11q24.2-q25, and LOH at 13q12-q13. B[a]P-induced cell transformation was also associated with LOH at 13q12-q13 and at 17p13.2. The relevance of these findings is highlighted by the observation that E₂- and B[a]P-induced genomic alterations in the same loci found in ductal hyperplasia, ductal carcinoma *in situ*, and invasive ductal carcinoma of the breast. Environ. Mol. Mutagen. 39:254–263, 2002. © 2002 Wiley-Liss, Inc.

Key words: breast cancer; neoplastic transformation; epithelial cells; estrogens; chemical carcinogens

INTRODUCTION

Breast cancer is the most frequently diagnosed malignancy in American women, with 182,800 cases detected and 40,800 deaths in the United States during the year 2000 [Greenlee et al., 2000]. Epidemiological and clinical evidence indicate that breast cancer risk is associated with prolonged exposure to female ovarian hormones [Bernstein and Ross, 1993; Kelsey et al., 1993; Pike et al., 1993; Greenlee et al., 2000]. Breast cancer is a hormone- and sex-dependent malignancy whose development is influenced by a myriad of hormones and growth factors [Topper et al., 1986; Henderson et al., 1988]. Estrogens have been demonstrated to be of essential importance in this disease because it is observed in postmenopausal hyperestrogenism resulting from the use of estrogenic hormone replacement therapy and obesity [Lippman et al., 1986; Russo et al., 2000]. Estrogens, which are necessary for the normal development of both reproductive and nonreproductive or-

gans, exert their physiological effects by binding to their specific receptors, the estrogen receptors ER α or ER β . Estrogens might act as well through alternate nonreceptor mediated pathways [Chen et al., 2000]. Passive and active exposure to tobacco smoke, which contains benzo[a]pyrene (B[a]P), is also considered an etiologic factor for breast cancer [Bennett et al., 2000; Wells, 2000]. The link between 17 β -estradiol (E₂) and B[a]P is that both are metabolically

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activated by xenobiotic metabolizing enzymes such as those of the cytochrome P450 family, which have been identified in lung and breast tissues [Hellmold, 1998].

Enzymatic activation of carcinogens yields intermediate metabolites that are chemically more reactive than the initial compound [Hellmold, 1998; Bennett et al., 2000; Cavalieri et al., 2000]. These enzymes contribute to polycyclic aromatic hydrocarbon (PAH)-dependent carcinogenesis by promoting production of DNA-reactive intermediates, such as diol epoxides [Hellmold, 1998; Cavalieri et al., 2000; Russo et al., 2000]. Among the P450 isoforms, it is known that both human and rat CYP1A1 metabolize endogenous E₂ and also activate the carcinogen B[a]P. Another isoform, CYP1B1, metabolizes estradiol to its carcinogenic metabolite 4-hydroxyestradiol and activates the polycyclic hydrocarbon 7,12-dimethylbenz[a]anthracene (DMBA). In addition, these two isoforms also activate other PAHs and heterocyclic amines [Hellmold, 1998; Jefcoate et al., 2000]. Other mechanisms that have been reported to play a role in determining the carcinogenic potential are alterations in the detoxification of carcinogenic compounds, which are mediated by the conjugation of the compounds or their metabolites to glutathione by glutathione S-transferase (GST). GSTs are class II detoxification enzymes that are encoded by four classes of polymorphic genes [Bennett et al., 2000].

Breast cancers exhibit genomic alterations, such as DNA amplification and loss of genetic material, that may represent tumor-suppressor genes [Chen et al., 1989, 1992, 1994; Genuardi et al., 1989; Sato et al., 1991, 1992, 1994; Russo et al., 1993a,b; Calaf et al., 1995], although the role of these genomic alterations in the causation of the disease has not yet been clearly established. Specific types of genetic alterations, then, might identify essential steps in the initiation and/or progression of cancer. We postulate that, if estrogens and chemical carcinogens such as B[a]P initiate the neoplastic process or are responsible for its progression, they would induce in the normal breast epithelium the same type of genomic alterations observed in spontaneous malignancies. To test this hypothesis we evaluated the transforming potential of E₂ and B[a]P on human breast epithelial cells (HBECs) *in vitro*, utilizing the spontaneously immortalized HBEC MCF-10F [Soule et al., 1990; Tait et al., 1990]. This cell line lacks both ER α and ER β , although this latter receptor is induced in cells transformed by chemical carcinogens [Hu et al., 1998]. The same phenotypes and characteristics that were expressed by MCF-10F cells transformed by the chemical carcinogens and oncogenes [Calaf and Russo, 1993; Russo et al., 1993a; Calaf et al., 1995] were evaluated in E₂- and B[a]P-treated cells: anchorage-independent growth, colony formation in agar methocel, and ductulogenic capacity in collagen gel. In addition, the DNA of treated cells was analyzed for specific genomic alterations such as loss of heterozygosity (LOH) at chromosomal loci known to be affected in spontaneously occurring breast lesions, such as ductal hyperplasia, ductal carcinoma *in situ*,

and invasive carcinoma [Theillet et al., 1986, 1990; Mackay et al., 1988; Takita et al., 1992; Winqvist et al., 1993, 1995; Carter et al., 1994; Chen et al., 1994; Bergthorsson et al., 1995; Gudmundsson et al., 1995; Negrini et al., 1995a, 1996; Kerangueven et al., 1996; Man et al., 1996; Koreth et al., 1997; Pandis et al., 1997].

THE TARGET CELL OF MAMMARY CARCINOGENESIS

The breast is a hormone-responsive organ *par excellence*. Its development is influenced by a myriad of hormones and growth factors, responding selectively to given hormonal stimuli with either cell proliferation or differentiation. Among all the complex hormonal influences, estrogens are considered to play a major role in promoting the proliferation of both the normal and the neoplastic breast epithelium. In humans, the highest level of cell proliferation is observed in the undifferentiated lobules type 1 (Lob 1) present in the breast of young nulliparous females [Russo et al., 2000]. The progressive differentiation of Lob 1 into Lob 2 and Lob 3, occurring under the hormonal influences of the menstrual cycle, and the full differentiation into Lob 4, as the result of pregnancy, lead to a concomitant reduction in the proliferative activity of the mammary epithelium [Russo et al., 2000]. Of interest is the fact that the content of ER α and PgR in the lobular structures of the breast is directly proportional to the rate of cell proliferation. These three parameters are maximal in the undifferentiated Lob 1, decreasing progressively in Lob 2, Lob 3, and Lob 4. ER α - and PgR-positive cells are found exclusively in the epithelium; the myoepithelium and the stroma are totally devoid of steroid receptor-containing cells. The highest number of cells positive for both receptors is found in Lob 1, decreasing progressively in Lob 2 and Lob 3 [Russo et al., 2000].

To clarify the relationship between steroid receptor-positive cells and proliferating cells we utilized a double-staining procedure, combining in the same tissue section anti-Ki67 and ER α , Ki67 and PgR, or ER α and PgR antibodies. It was found that a higher percentage of cells reacted simultaneously with both ER α and PgR, appearing purplish red in color, whereas the number of cells positive for both ER α and Ki67 or PgR and Ki67 was very low. The highest percentage of ER α -, PgR-, and Ki67-positive cells was observed in Lob 1. The percentages of Ki67-, ER α -, and PgR-positive cells were reduced in Lob 2 and became negligible in Lob 3. The utilization of a double-labeling immunocytochemical technique has allowed us to demonstrate that the expression of the receptors occurs in cells other than the proliferating cells, confirming results reported by others. The findings that proliferating cells are different from those that are ER α - and PgR-positive support data indicating that estrogen controls cell proliferation by an indirect mechanism. This phenomenon has been demonstrated using the supernatant of estrogen-treated ER α -pos-

itive cells that stimulates the growth of ER α -negative cell lines in culture. The same phenomenon has been shown in vivo in nude mice bearing ER-negative breast tumor xenografts. ER α -positive cells treated with antiestrogens secrete TGF β that inhibits the proliferation of ER α -negative cells. The fact that the highest proliferative activity and the highest percentages of ER α - and PgR-positive cells are present in Lob 1 provides a mechanistic explanation for the higher susceptibility of these structures to be transformed by chemical carcinogens in vitro, supporting as well the observation that Lob 1 is the site of origin of ductal carcinomas [Russo et al., 2000].

IN VITRO MODEL OF CELL TRANSFORMATION

General Concepts

It is generally accepted that malignant transformation involves genetic and epigenetic changes that derail common regulatory mechanisms, resulting in uncontrolled cellular proliferation and/or aberrant programmed cell death or apoptosis [Russo et al., 1988, 1993a,b, 1996, 1998; Holliday, 1996]. These cellular abnormalities, hallmarks of a carcinogenic process, are frequently associated with molecular alterations involving activation of protooncogenes and inactivation of tumor-suppressor genes as a result of genetic predisposition and/or exposure to physical (e.g., radiation), chemical (e.g., carcinogens, dietary components), and biological (e.g., viruses) environmental factors [Briand et al., 1987; Russo et al., 1988, 1993a,b, 1996, 1998; Band et al., 1990; Bartek et al., 1990; Soule et al., 1990; Tait et al., 1990; Garcia et al., 1991; Calaf and Russo, 1993; Couch 1996; Holliday, 1996; Hu et al., 1997]. A central challenge to cancer biology is the understanding of the cellular and molecular processes that drive a normal human breast epithelial cell to neoplastic growth. In vitro models have proven to be useful for testing whether chemical carcinogens can be causative agents of breast cancer and whether genomic changes play a functional role in the initiation and progression of this disease. HBECs are susceptible to undergoing neoplastic transformation when treated with estrogens or B[a]P. Transformation of HBECs in vitro, however, requires that specific conditions be met by the target cells, similar to what has been observed in in vivo experimental models [Russo et al., 1993a,b, 1996]. Only under optimal conditions of susceptibility will specific carcinogens initiate a cascade of changes in HBECs that recapitulate the phenotypic stages of tumor initiation and progression, culminating in the expression of tumorigenesis in a heterologous host [Calaf and Russo, 1993].

Transformation of Breast Epithelial Cells

Normal HBECs senesce after 10–20 passages in vitro when cultured in standard culture medium containing 1.05

mM calcium (Ca $^{++}$) [Soule et al., 1990; Tait et al., 1990]. We have developed an in vitro system that has allowed us to determine that the ability of HBECs to grow in culture greatly reflects their in vivo characteristics, that is, the degree of lobular development of the breast tissues from which they were obtained. Lobular development, which in turn determines the epithelium's rate of proliferation, is the final result of life-time influences, such as aging, endocrine, reproductive, and environmental factors [Russo et al., 1988, 1996]. These characteristics influence as well the response of the cells to the transforming potential of chemical carcinogens known to be of etiologic importance in various experimental models of mammary cancer [Russo et al., 1993a,b]. When primary cultures of HBECs obtained from Lob 1, which represent the most undifferentiated structure present in the breast of young nulliparous females, and from the more differentiated Lob 3 of parous women were placed in culture, it was observed that cells obtained from Lob 1 retained in vitro a higher rate of cell proliferation and exhibited greater survival efficiency than cells obtained from Lob 3 [Russo et al., 1988, 1993a,b, 1996].

Treatment of the cells with the chemical carcinogens 7,12-dimethylbenz[a]anthracene (DMBA), 1-methyl-3-nitro-1-nitroso-guanidine (MNNG), *N*-methyl-*N*-nitrosourea (NMU), or B[a]P increased survival efficiency in cells from Lob 1, but full transformation was not achieved. These results indicated that the susceptibility of HBECs in vitro was influenced by the differentiation status of the breast in vivo [Russo et al., 1988, 1993a]. However, the fact that primary cell cultures only expressed increased survival efficiency, an early phenotypic marker of neoplastic transformation, indicated that further elucidation of the conditions required for inducing cell transformation with chemicals was needed. The spontaneous immortalization of the HBEC line MCF-10M, derived from the human breast tissue sample number 130 (S130), provided such conditions [Soule et al., 1990; Tait et al., 1990]. After 2 years of continuous culture of the mortal MCF-10M cells in medium containing a 0.04 mM Ca $^{++}$ (low Ca $^{++}$), they spontaneously gave rise to an immortal cell line, MCF-10, which grew either as attached (MCF-10A) or as floating (MCF-10F) cells [Soule et al., 1990; Tait et al., 1990]. Immortalization of these cells was characterized by their continuous growth in culture medium containing the conventional 1.05 mM calcium concentration (that for our purposes was called "high Ca $^{++}$ medium") without entering into senescence [Soule et al., 1990; Tait et al., 1990]. The analysis of the growth characteristics of these primary cells and the cell lines derived from them revealed that the growth curves of MCF-10M, MCF-10A, and MCF-10F cells were similar, independent of the calcium concentration in the culture medium. Mortal MCF-10M cells, however, were unable to continue growing in high Ca $^{++}$ medium after the 20th passage, whereas the immortal cells MCF-10A and MCF-10F continued growing indefinitely. The human breast epithelial origin of the mor-

tal and the immortalized cells was confirmed by their genetic, cytogenetic, ultrastructural, and phenotypic characteristics [Soule et al., 1990; Tait et al., 1990]. The immortal MCF-10F cells were identical to the mortal MCF-10M cells from which they were derived in all the aspects for which they were evaluated. The only difference found to date is that MCF-10F cells are pseudodiploid and express minimal chromosomal alterations (46XX, 1p+, t[3;9][p13;p22]) [Soule et al., 1990; Tait et al., 1990; Russo et al., 1996].

Dose-Response Effect of E_2 in HBECs

To determine the optimal doses for the expression of the cell transformation phenotype, we treated the immortalized HBEC MCF-10 F with E_2 for testing the survival efficiency (SE), colony-forming ability in agar methocel, or colony efficiency (CE) and loss of ductulogenesis in collagen matrix. MCF-10F cells were treated with 0.0, 0.007 nM, 70 nM, or 0.25 mM of E_2 twice a week for 2 weeks [Russo et al., 2001]. The SE was increased with 0.007 and 70 nM of E_2 and decreased with 0.25 mM. The cells treated with either doses of E_2 formed colonies in agar methocel that did not differ in size; however, the CE increased from 0 in controls to 6.1, 9.2, and 8.7 with increasing E_2 doses. Ductulogenesis, or the number of ductules per 10,000 cells plated, was 75 ± 4.9 in control cells; it decreased to 63.7 ± 28.8 , 41.3 ± 12.4 , and 17.8 ± 5.0 in E_2 -treated cells, which also formed spherical-like structures or solid masses, whose numbers increased from 0 in controls to 18.5 ± 6.7 , 107 ± 11.8 , and 130 ± 10.0 for each E_2 dose.

Estrogen and Chemical Carcinogens Induce Transformation Phenotypes

Evaluation of colony formation at the end of the second week of E_2 and B[a]P treatment revealed that MCF-10F cells formed colonies in agar methocel over 60 microns in diameter. MCF-10F control cells treated with DMSO did not form colonies. The total CE was significantly increased by E_2 and B[a]P (Figs. 1 and 2).

Ductulogenesis was qualitatively evaluated by estimating the ability of the cells plated in collagen to form ductular structures (cells lining a lumen). It was maximal in MCF-10F cells (Fig. 3a) and completely negative (−) in B[a]P-treated cells, which grew as a solid or cystic mass. All the cells treated with E_2 exhibited decreased ability to form ductules (Fig. 3b–e). Progesterone did not significantly affect the ductulogenic capacity. The collagen matrix embedded in paraffin and cross-sectioned for determination of cell morphology showed that MCF-10F cells form a well-defined ductule lined by a monolayer of cuboidal epithelial cells (Fig. 4a), whereas with those treated with E_2 , the number of layers increase and in some cases the whole lumen is obliterated (Fig. 4b–d). B[a]P also forms structures similar to those induced by estrogen, whereas the ductules

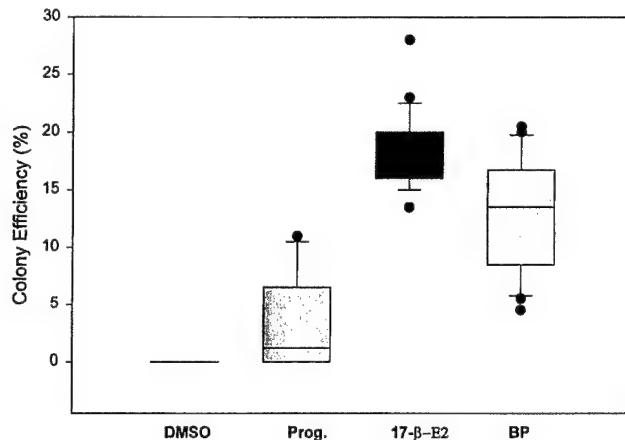


Fig. 1. Box plot showing the effect of different compounds on MCF-10F cell colony efficiency in agar methocel.

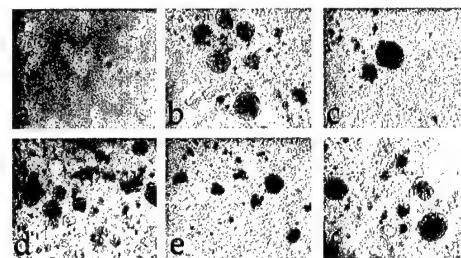


Fig. 2. MCF-10F cells plated in agar methocel for colony assay. a: Control cells do not form colonies, only isolated cells are present. b–d: Colonies formed by E_2 -treated MCF-10F cells at the doses of 0.007 nM (b), 70 nM (c), and 1 μ g (d). e: Progesterone-treated cells. f: B[a]P-treated cells induce slightly larger colonies. Phase-contrast microscope: $4 \times$ magnification.

formed by progesterone treatment are smaller, with a reduced luminal size lined by a monolayer of cuboidal epithelial cells.

Genomic Changes Induced in E_2 -Transformed Cells

From the E_2 -treated cells, six clones out of 24 colonies were expanded and maintained in culture. These clones, designated E_2 -1 to E_2 -6 (Table I), were selected for genomic analysis. DNA fingerprint analysis of parent E_2 - and B[a]P-treated cells and their derived clones revealed that their allelic pattern was identical in all the cell lines analyzed. These results confirmed that all the cells tested had the same HBEC origin and that they were free of contamination from other cell lines maintained in our laboratory. Among 67 markers tested, which were selected based on chromosomal changes reported to be present in breast and other cancers, only clones E_2 -1 and E_2 -2 identically exhibited LOH in chromosome 11 at 11q23.3 (marker *D11S29*) and 11q24.2–q25 (marker *D11S912*). B[a]P-treated cells did not exhibit LOH at any of the loci tested for chromosome 11. Interestingly, we have found that all the clones of the cells trans-

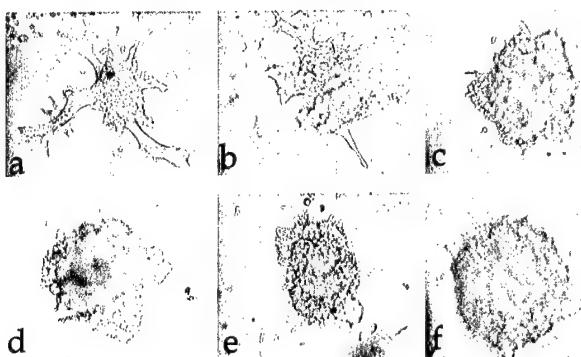


Fig. 3. **a:** MCF 10F cells treated with solvent (DMSO) forming well-defined ductular structures in collagen matrix; **(b)** 0.007 nM of E₂ induces alteration in the ductular pattern; **(c, d)** 70 nM of E₂ induces the loss of ductular formation in collagen matrix; **(e, f)** 1 µg of E₂ or B[a]P, respectively, induces the formation of spherical masses in collagen matrix. Phase-contrast microscope: 10 × magnification.

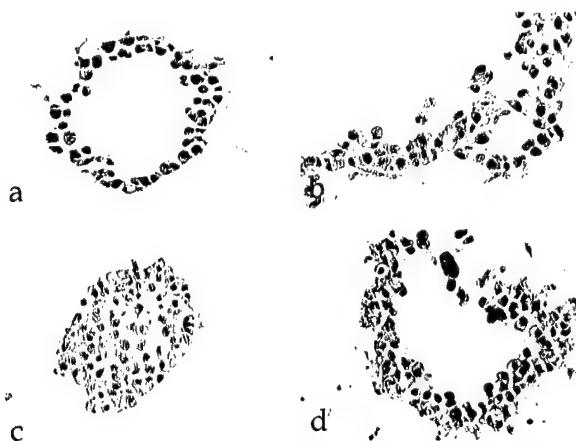


Fig. 4. Histological sections of cells growing in collagen gel. The cells have been fixed in buffered formalin, embedded in paraffin and the sections stained with hematoxylin and eosin. **a:** MCF 10F cells treated with solvent (DMSO) forming well-defined ductular structures lined by a single cuboidal layer of cells; **(b)** 0.007 nM of E₂ induces alteration in the ductular pattern forming spherical masses lined by two to three layers of cells; **(c)** 70 nM of E₂ induces the loss of ductular formation in collagen matrix and the solid spherical masses are composed of large cuboidal cells; **(d)** 1 µg of E₂ or B[a]P induces the formation of spherical masses lined by multiple layers of cells. Bright field: 10 × magnification.

formed with either E₂ or B[a]P had microsatellite instability (MSI), expressed as an allelic expansion at the 3p21 locus (marker D3S1447) (data not shown). It was of great interest that by the fourth passage after four treatments during a 2-week period, clones derived from E₂-transformed cells exhibited LOH in chromosome 11. It was previously reported that both arms of chromosome 11 contain several regions of LOH in cancers of the breast and of other organs, and that transfer of chromosome 11 to mammary cell lines suppresses tumorigenicity in athymic mice [Negrini et al., 1995].

Several genes, such as *HRAs*, *CTSD*, *ILK*, *TSG101*, and

KII have been located to the short arm of chromosome 11 [Theillet et al., 1986; Mackay et al., 1988; Puech et al., 1991; Borresen et al., 1992; Negrini et al., 1994; Dong et al., 1995; Hannigan et al., 1997; Wang et al., 1997]. A region of deletion on 11q22–23 has been described on the long arm of chromosome 11 in 40–60% of breast tumors [Carter et al., 1994; Hampton et al., 1994; Negrini et al., 1995; Winqvist et al., 1995; Man et al., 1996; Hannigan et al., 1997; Koreth et al., 1997]. The ataxia telangiectasia susceptibility gene (*ATM*) is the most widely studied candidate gene in this region [Elson et al., 1996]. *ATM* may act upstream of the *TP53* gene in cell cycle regulation [Swift et al., 1991; Westphal et al., 1997], and its heterozygous mutation is associated with a high incidence of early-onset breast cancer. This region has been reported to contain several tumor-suppressor genes and genes involved in the metastatic process. In this latter group, the *MMP* genes encoding matrix metalloproteases involved in invasion, *ETS1*, encoding a transcription factor involved in angiogenesis, and *VACM-1*, encoding a protein probably involved in cell cycle regulation, have been identified [Swift et al., 1991; Byrd et al., 1997]. Although some of these genes might be affected during the transformation of HBECs by estrogens, a more detailed allelotyping using multiple markers is required for better defining the size of the region exhibiting LOH in these cells. Approximately 35% of breast cancers have LOH at the *D11S29* and *NCAM* loci [Tomlinson et al., 1996a], and a higher frequency of LOH at this locus has also been found in melanomas [Tomlinson et al., 1996b]. LOH has been found at frequencies of 25% and 29% at the distal *D11S968* (11qter) and *D11S29* (11q23.3 locus), slightly above the accepted baseline of 0–20% in colorectal cancer. The fact that breast cancer, melanoma, and colorectal cancer are influenced by estrogens [Connolly et al., 1999] gives relevance to our data that treatment of MCF-10F cells with estrogens induces LOH in this specific locus. LOH at 11q23–qter occurs frequently in ovarian and other cancers [Dahiva et al., 1997; Launonen et al., 1998].

Genomic Changes Induced in Chemically Transformed Cells

We utilized DNA amplification of microsatellite length polymorphisms for detecting whether either allelic loss or microsatellite instability was present in MCF-10M, MCF-10F, BP1, BP1E, D3, and D3-1 cells at different passages [Huang et al., 1999; Yang et al., 1999]. Of all the cell lines and clones tested, only BP1E cells exhibited LOH in chromosome 17p13, which was detected first with the *P144D6* marker [Wu et al., 1997; Russo et al., 1998] and later on narrowed using the marker *D17S796* [Lareef et al., 2001].

The expression of transformation phenotypes was preceded by microsatellite instability, as revealed by the observation that the transformed BP1 and BP1E cells, which expressed anchorage independence, loss of ductule-like for-

TABLE I. Phenotypic Markers of Cell Transformation Induced in MCF-10F Cells by 17 β -Estradiol (E₂) and Benzo[a]pyrene (B[a]P)

Cell type	No. of passages	Doubling time (DT) ^a	Colony number (CN) ^b	Colony efficiency (%) (CE) ^c	Colony size (CS) (μ m) ^d
<i>MCF-10F</i>	113	93 \pm 5.6	0.0	0.0	0.0
<i>B[a]P</i> -derived	4	42 \pm 3.8	89	18 \pm 4.5	670 \pm 46
E ₂ -derived	4	78 \pm 16.0	24 ^e	4.8 \pm 0.9	170 \pm 34
E ₂ -1 ^f	4	81 \pm 3.0	36	7.2 \pm 3.7	180 \pm 12
E ₂ -2 ^f	4	68 \pm 10	45	9.0 \pm 2.0	150 \pm 6
E ₂ -3	5	66 \pm 8.0	39	7.9 \pm 5.6	190 \pm 9
E ₂ -4	3	82 \pm 6.0	20	3.5 \pm 1.1	134 \pm 5
E ₂ -5	6	61 \pm 5.6	63	12.6 \pm 3.0	193 \pm 12
E ₂ -6	4	73 \pm 3.0	54	10.8 \pm 4.9	189 \pm 5

^aDoubling time (DT) in hr (expressed as mean \pm SD) was determined as described in Calaf and Russo [1993]. DT was significantly different by Students' t-test between B[a]P-treated and all other cell lines ($P < 0.001$).

^bColony number (CN) was significantly different between MCF-10F and all other cell lines ($P = 0.00001$).

^cColony efficiency (CE) (expressed as mean \pm SD) was significantly different between MCF-10F and all other cell lines ($P = 0.00001$).

^dColony size (CS) (expressed as mean \pm SD) was significantly different between MCF-10F and all other cell lines ($P = 0.00001$). CS of DES clones was significantly different from E₂- and B[a]P-treated cells ($P = 0.001$).

^eFrom 24 colonies derived from E₂-treated cells, clones E₂-1, E₂-2, E₂-3, E₂-4, E₂-5, and E₂-6 were recovered and expanded.

^fThese cells have been used for detection of microsatellite DNA polymorphism.

mation in collagen gel, and increased chemotactic and invasive properties, also expressed MSI on chromosome 11 at loci represented by *D11S912* at q25 and on chromosome 13 at 13q12-13 (flanking the *BRCA2* locus), as detected with markers *D13S260* and *D13S289* [Huang et al., 1999]. MSI in chromosome 11 at 11p13 and chromosome 17 at 17p13.3 and 17p13.1 was also present, although they were retained from the parent immortalized MCF-10F cells, as was a variant band in one of the heterozygous alleles of *TP53* of MCF-10F cells, which was detected using single-strand conformational polymorphism [Huang et al., 1999]. D3 and D3-1 cells, which expressed an early stage of transformed phenotype equivalent to that of the BP1 cells, also exhibited MSI at locus *D13S260* on chromosome 13q12-13 [Huang et al., 1999] and in *D16S285* on chromosome 16q12.1 [Wu et al., 1997]. D3 and D3-1 cells did not exhibit as many genetic alterations as the B[a]P-derived cell lines [Huang et al., 1999]. Neoplastic progression was also associated with mutations and/or amplification of *c-H-ras*, *int-2*, *c-neu*, *c-myc*, and *MDM2* genes [Wu et al., 1997; Russo et al., 1998], in addition to MSI at 11q25 and 13q12-q13 and LOH at 17p13.2. Detection of MSI in our in vitro model served as an indicator of the progression of normal HBECs from immortalization to transformation, a phenomenon initiated and driven by the carcinogen. The relevance of these findings is highlighted by the observation that the loci in which MSI and LOH were detected in the HBECs transformed with chemical carcinogens are the same that are found in primary breast cancers, as described below.

Genomic Changes in Human Breast Lesions

Microsatellites, which are short, repetitive sequences of DNA scattered throughout the genome [Weber and May,

1989; Boyer et al., 1995] have been used to detect genomic alterations in various human cancers through the identification of LOH or MSI [Ionov et al., 1993; Wooster et al., 1994]. Studies using this approach provided direct evidence for the involvement of chromosome 13 in human breast carcinomas and led to the identification of the well-known tumor-suppressor gene *RB1* (13q14) and breast cancer susceptibility gene *BRCA2* (13q12-13) [Wooster et al., 1998]. We previously observed MSI at the chromosomal regions 13q12-13, 11q25, and 16q12.1 in the early stages of chemical transformation of HBECs [Wu et al., 1997; Huang et al., 1999]. We tested the hypothesis that, if MSI represents an early event in breast carcinogenesis *in vivo*, a relationship exists between microsatellite alteration and the progression of cancer. Thus, we performed polymorphic studies on ductal carcinoma *in situ* (DCIS) of the breast using three microsatellite DNA polymorphic markers, *D13S289*, *D13S260*, and *D13S267*, that flank the *BRCA2* region at 13q12-13.

Paraffin-embedded breast tumors were obtained from the patient sample depository at the Breast Cancer Research Laboratory, Fox Chase Cancer Center (Philadelphia, PA). Microscopically identifiable populations of epithelial cells from normal ducts and DCIS were collected by microdissection from 10 μ m-thick serial paraffin sections [Aldaz et al., 1995; Radford et al., 1995]. Paraffin sections were deparaffinized with xylene, rehydrated, and digested with 0.15 μ g of proteinase K followed by organic extraction. The DNA was precipitated with ethanol, resuspended in TE, and stored at 4°C until use. Microsatellite DNA was amplified by PCR [Huang et al., 1999]. MSI was defined as an increase or decrease in the number of bands and/or in the size of one or both alleles in relation to the normal alleles [Aaltone et al., 1993; Thibodeau et al., 1993]. Of 35 infor-

TABLE II. Expression of Microsatellite Instability (MSI) in Human Breast Epithelial Cells Treated With Chemical Carcinogens in Vitro

Sites of MSI	Cell line					
	MCF-10M	MCF-10F	BP1	BP1-E	D3	D3-1
Marker (locus)		<i>D11S1392</i> (11p13)	<i>D11S1392</i> (11p13)	<i>D11S1392</i> (11p13)		
Marker (locus)			<i>D11S912</i> (11q25)	<i>D11S912</i> (11q25)		
Marker (locus)			<i>D13S260</i> (13q12-13)		<i>D13S260</i> (13q12-13)	<i>D13S260</i> (13q12-13)
Marker (locus)			<i>D13S289</i> (13q12-13)			
Marker (locus)	<i>D17S849</i> (17p13.3)	<i>D17S849</i> (17p13.3)	<i>D17S849</i> (17p13.3)	<i>D17S849</i> (17p13.3)	<i>D17S849</i> (17p13.3)	<i>D17S849</i> (17p13.3)
Marker (locus)	<i>D17S796</i> (17p13.1)	<i>D17S796</i> (17p13.1)	<i>D17S796</i> (17p13.1)	<i>D17S796</i> (17p13.1)	<i>D17S796</i> (17p13.1)	<i>D17S796</i> (17p13.1)
Marker (locus)	<i>D17S513</i> (17p13.1)	<i>D17S513</i> (17p13.1)	<i>D17S513</i> (17p13.1)	<i>D17S513</i> (17p13.1)	<i>D17S513</i> (17p13.1)	<i>D17S513</i> (17p13.1)
Marker (locus)	<i>Tp53</i> (17p13.1)	<i>Tp53</i> (17p13.1)	<i>Tp53</i> (17p13.1)	<i>Tp53</i> (17p13.1)	<i>Tp53</i> (17p13.1)	<i>Tp53</i> (17p13.1)
Marker (locus)	<i>D17S786</i> (17p13.1)	<i>D17S786</i> (17p13.1)	<i>D17S786</i> (17p13.1)	<i>D17S786</i> (17p13.1)	<i>D17S786</i> (17p13.1)	<i>D17S786</i> (17p13.1)

mative DCIS, MSI was found to be present in 5 (14%) and LOH in 4 (11%) of the cases with marker *D13S260*. At locus *D13S267*, which is more distal to *D13S260*, MSI was seen in 3 (9%) and LOH in 8 (24%) of a total of 33 informative cases. Marker *D13S289*, the most distal of those flanking the *BRCA2* locus had 23 informative cases, 5 of which were affected by MSI (22%) and 2 by LOH (9%) (Table II). Nine (30%) of these cases exhibited MSI and LOH at loci *D13S260*, 9 (26%) at *D13S267*, and 7 (30%) at *D13S289*, indicating that these three markers reflected approximately the same incidence of genomic alterations at the *BRCA2* region.

The application of microsatellite DNA polymorphic analysis provided evidence that MSI and LOH in chromosomal region 13q12-13 are associated with the development of preinvasive breast cancers, thus indicating the involvement of DNA repair defects and/or gene inactivation in breast carcinogenesis. To further test the pattern of expression of genomic changes in the progression of breast cancer, we performed microsatellite polymorphism analysis in genomic DNA extracted by microdissection from breast tissues of 21 breast cancer patients that contained three different types of breast lesions: ductal hyperplasia (DHP), DCIS, and invasive ductal carcinoma (INV) (Table III). We analyzed specific loci on chromosomes 11, 13, 16, and 17 using an array of 7 markers, *D11S912*, *D11S940*, *D13S260*, *D13S289*, *D13S267*, *D16S285*, and *D17S855*. Among these markers, *D11S912* showed MSI in 10/19 (53%) of all samples including 2/8 of the preneoplastic lesion DHP, 8/15 DCIS, and 3/5 of INV (Table III). MSI was detected in chromosome 13 with marker *D13S260* in 5/16 (31%) DCIS and 3/4 (75%) INV (Table III), but was absent in DHP, suggesting a correlation with the progression of the disease, and con-

TABLE III. Microsatellite Instability (MSI) and Loss of Heterozygosity (LOH) in Ductal Carcinoma in Situ of the Breast

Marker	Map Position	Total cases ^a	MSI (%)	LOH (%)	MSI and LOH (%)
<i>D13S260</i>	13q12-13	35	5 (14)	4 (11)	9 (26)
<i>D13S267</i>	13q12-13	33	3 (9)	8 (24)	11 (33)
<i>D13S289</i>	13q12-13	23	5 (22)	2 (9)	7 (30)

^aIncludes both informative and uninformative cases.

sistent with its alteration in more advanced phases of in vitro transformation, such as in BP1E cells. In addition, the high MSI incidence in all samples for markers *D11S912* (53%), *D13S260* (23%), *D13S289* (36%), and *D13S267* (45%), compared to lower rates of *D11S940* (10%), *D16S285* (6%), and *D17S855* (9%), may suggest that instability preferentially occurs in specific loci during breast carcinogenesis, also in good agreement with the data from the transformed HBEC system (except for *D13S267*).

In conclusion, the observation that *E₂*, B[a]P, and other chemical carcinogens induce similar phenotypic changes, but different genomic alterations in vitro, could be an indication of different genomic pathways for transformation. Interestingly, the data show that in both cases the genomic alterations detected are the ones that are also observed in breast lesions. These observations support the premise that substances that are carcinogenic to the HBECs and responsible for the initiation of the neoplastic process induce the same type of genomic alterations in the normal breast epithelium that are observed in spontaneous malignancies.

TABLE IV. Microsatellite Instability (MSI) and Loss of Heterozygosity (LOH) in Ductal Hyperplasia (DHP), Ductal Carcinoma in Situ (DCIS), and Invasive Carcinoma (INV)

Marker	Map Position	Histopathological type of breast lesions					
		DHP ^a		DCIS ^b		INV ^c	
		MSI	LOH	MSI	LOH	MSI	LOH
<i>Int2</i>	11q13.3	2/24 (8.3)	0/24 (0)	4/35 (11.4)	2/35 (5.7)	2/14 (14.3)	0/14 (0)
<i>D11S614</i>	11q21-23.3	2/33 (6.1)	0/30 (0)	3/38 (7.9)	0/38 (0)	2/16 (12.5)	0/16 (0)
<i>D11S912</i>	11q25	3/37 (5.4) ^b	1/33 (3.03) ^c	15/54 (27.8) ^b	5/54 (9.2) ^c	3/22 (13.6) ^b	8/22 (36.4) ^c
<i>D11S940</i>	11q21-23.3	1/27 (3.7)	0/37 (0)	2/39 (5.1)	1/39 (2.6)	2/16 (12.5)	0/16 (0)
<i>D13S260</i>	13q12-13	0/14 (0)	0/14 (0)	3/23 (13)	3/23 (13)	2/13 (15)	4/13 (31)
<i>D13S267</i>	13q12-13	0/11 (0)	0/11 (0)	3/21 (14)	7/21 (33)	1/12 (8)	2/12 (17)
<i>D13S289</i>	13q12-13	0/7 (0)	0/7 (0)	3/11 (27)	2/11 (18)	1/5 (20)	3/5 (60)

^aNo. lesions affected/no. informative cases (%).^bFisher's exact test, $P < 0.05$.^cFisher's exact test, $P < 0.01$.

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